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TABLE OF CONTENTS

Vol. XXV. 1937-38.

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No. 1 (July 1937).

	PAGE
AYKROYD, W. R., and KRISHNAN, B. G. A Diet Survey repeated at another Season	1
AYKROYD, W. R., and WRIGHT, R. E. Red-Palm Oil in the Treatment of Human Keratomalacia	7
DE, N. K. The Possible Use of Red-Palm Oil in supplementing the Vitamin-A Activity of common Vegetable Oils	11
DE, N. K. The Adsorption of Vitamin A and Carotene and the Isolation of Vitamin A from associated Pigments	17
RANGANATHAN, S., and SANKARAN, G. The Excretion of Vitamin C by Human Beings in South India	29
RADHAKRISHNA RAO, M. V. Studies on Vitamin-A Deficiency. Part II. Histopathology of the Skin in Human Keratomalacia	39
RANGANATHAN, S., SUNDARARAJAN, A. R., and SWAMINATHAN, M. Survey of the Nutritive Value of Indian Foodstuffs. Part II. Changes in Chemical Composition brought about by Cooking	45
SWAMINATHAN, M. The Relative Value of the Proteins of certain Foodstuffs in Nutrition. Part II. The Comparative Biological Values of the Proteins of certain Cereals, Pulses, and Skimmed Milk Powder, measured by the Growth of young Rats	57
SANKARAN, G., and RANGANATHAN, S. Migration of Adrenaline in an Electrical Field	81
WILSON, H. E. C., AHMAD, B., and MAJUMDAR, B. N. The Transformation of Carotene into Vitamin A in Liver Autolysates	85
RUDRA, M. N. Studies in Vitamin C. The Effect of Cooking and Storage on the Vitamin-C Contents of Foodstuffs	89
KRISHNAN, B. T. Influence of Histamine and Acetyl-Choline on Intestinal Movements	95

	PAGE
CHOPRA, R. N., and DE, N. N. A Preliminary Note on the Presence of a Histamine-like Body in the Aqueous Humour in Glaucoma associated with Epidemic Dropsy	101
CHOPRA, R. N., and ROY, A. C. Observations on the Blood-Lipoid Changes in Opium Addicts	105
MUKERJI, B. Action on Smooth Muscles of some Ephedrine-like Amines ..	113
HARDIKAR, S. W., and MOHIUDDIN, M. G. Chemical and Pharmacological Examination of <i>Randia dumetorum</i> , N. O. <i>Rubiaceæ</i>	131
CHOPRA, R. N., MUKHERJEE, S. N., and CHOWHAN, J. S. Physical Changes in Blood <i>in vivo</i> after Injection of Venom from Indian Cobra (<i>Naja naia vel tripudians</i>) into Monkeys	137
BAGCHI, K. N., and GANGULY, H. D. Lead in Urine and Fæces ..	147
MAPLESTONE, P. A., and BHADURI, N. V. <i>Tænia solium</i> and <i>Cysticercus cellulosæ</i> in India	155
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part I. Introductory Notes and a Historical Survey ..	163
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part II. A Summary of Field Studies	177
LAL, R. B., ROY, S. C., and GHOSAL, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part III. Some Laboratory Studies on Rice as the Ætiological Agent	215
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part IV. Experiments to test the Validity of Infection Theory in a Semi-isolated Community	233
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part V. Feeding Experiments on Human Volunteers	239
CHOPRA, R. N., DAS, N. N., and MUKHERJEE, S. N. Studies on the Ætiology of Epidemic Dropsy. Effect of Plasma on Tissue Culture and Chorionallantoic Membranes of the Chick	261
GALSTAUN, G. A Study of Ossification as observed in Indian Subjects ..	267

No. 2 (October 1937).

BASU, C. C. Notes on a New Strain of Actinomyces obtained by Blood Culture	325
JOHN LOWE and DHARMENDRA. A Study of <i>M. lepræ muris</i> in Tissue Cultures and in Chick-Embryo Medium	329
SHORTT, H. E., and SWAMINATH, C. S. The Viability of <i>Leishmania donovani</i> excreted in the Nasal Mucus in Indian Kala-azar	341

	PAGE
SMITH, R. O. A., and MERTA, D. R. Studies on Typhus in the Simla Hills. Part VII. Attempts to Isolate a Strain of XK Typhus from Wild Rats	345
MEHTA, D. R. Studies on Typhus in the Simla Hills. Part VIII. Ectoparasites of Rats and Shrews with Special Reference to their Possible Role in the Transmission of Typhus	353
AYKROYD, W. R., and KRISHNAN, B. G. The Deficiencies of the South Indian Diet	367
NARASIMHAMURTHY, G., and RANGANATHAN, S. The Nitrogen Complex of Indian Foodstuffs. Condiments: Part I. Black Pepper (<i>Piper nigrum</i>)	373
SWAMINATHAN, M. The Relative Value of the Proteins of certain Foodstuffs in Nutrition. Part III. The Biological Value of the Proteins of various Pulses, Oil-seeds, Nuts, and Skimmed Milk, studied by the Balance-sheet Method	381
SWAMINATHAN, M. The Relative Value of the Proteins of certain Foodstuffs in Nutrition. Part IV. Supplementary Values: Studied by (a) the Balance-sheet Method, using Adult Rats, and (b) the Growth Method, using Young Rats	399
DASTUR, N. N., and GIRI, K. V. Relative Digestibilities of Edible Fats by Castor-Seed and Pancreatic Lipases	427
GIRI, K. V. The Stabilization of Vitamin C by Pyrophosphate ..	443
CHOPRA, R. N., and ROY, A. C. On the Estimation of Atebrin in Tissues ..	455
CHOPRA, R. N., and BASU, B. C. A Preliminary Report on the Effect of Anti-Malarial Drugs upon the Infectivity of the Patients to the Mosquitoes	459
DE, S. P., and BASU, U. P. Action of p-Amino-benzene-sulphonamide against Streptococcal Infections in Mice	465
GHOSH, B. N., and RAY, N. N. Adsorption of Antigens by Antibodies or vice versa. Part IV. The Effect of different Factors on the Flocculation of Concentrated Antitoxins of Tetanus and Diphtheria in the Presence of their Respective Toxins	471
GHOSH, B. N., and RAY, N. N. The Danysz Phenomena in Staphylococcal Toxin-Antitoxin Reaction	477
SHORTT, H. E., MCGUIRE, J. P., BROOKS, A. G., STEPHENS, E. D., and LAHIRI, B. N. The Relative Immunizing Values of different Forms of Antirabic Vaccine, and the Duration of Immunity in Experimentally Immunized Animals	483
SWAMINATH, C. S., and SHORTT, H. E. The Arthropod Vector of <i>Babesia gibsoni</i>	499

	PAGE
SOKHEY, S. S., GOKHALE, S. K., MALANDKAR, M. A., and BILLIMORIA, H. S. Red Cells, Hæmoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part I. Normal Indian Men: A Study based on the Examination of 121 Men	505
NAPIER, L. E., and BILLIMORIA, H. S. Hæmatological Studies in Indians. Part VIII. Analysis of the Hæmatological Findings in 52 Cases of Anæmia amongst Pregnant Tea-garden Coolie Women	529
AHMAD, B., and GUHA, R. C. Some Observations on the Experimental Production of Cataract	547
SHORTT, H. E., McROBERT, G. R., BARNARD, T. W., and MANNADI NAYAR, A. S. Endemic Fluorosis in the Madras Presidency	553

No. 3 (January 1938).

LINTON, R. W., SHRIVASTAVA, D. L., and SEAL, S. C. Studies on the Specific Polysaccharides of the Vibrios. Part I. The Effect of the Growth- Medium	569
LINTON, R. W., SEAL, S. C., and MITRA, B. N. Chemical and Serological Variation in Single-Cell Cultures of <i>Vibrio cholerae</i> and Related Organisms	575
VENKATRAMAN, K. V., and PANDIT, C. G. An Epidemic of Cholera in a Rural Area in South India caused by the 'Ogawa' Type of <i>V. cholerae</i>	585
STEWART, A. D., and GHOSAL, S. C. On the Value of Wilson and Blair's Bismuth Sulphite Medium in the Isolation of <i>Bact. typhosum</i> from River Water	591
PANDIT, C. G., SANJIVA RAO, R., and SHORTT, H. E. Response of the Chorio-allantoic Membrane of the Developing Chick-Embryo to Inoculation with various Substances with Special Reference to <i>B. proteus</i> X19	595
MAPLESTONE, P. A., and DEY, N. C. Laboratory Tests on the Fungistatic and Fungicidal Effect of various Substances	603
RAY, N. N., and DAS, G. C. The Immunization of Horses for the Production of High-Titre Tetanus Antitoxin	617
GREWAL, K. S., and KOCHHAR, B. D. Vitamin-A Contents of Ghee	623
PATWARDHAN, V. N., and CHITRE, R. G. Studies in Calcium and Phosphorus Metabolism. Part I. The Calcium and Phosphorus Content of the Soft Tissues of Normal Rats	633
AYKROYD, W. R., and KRISHNAN, B. G. The Treatment of Stomatitis caused by Diet Deficiency	643
KRISHNAN, B. G. Skimmed Milk and the Growth of School Children	647

Table of Contents

ix

	PAGE
SHOURIE, K. L., and NARINDRA SINGH. A Diet Survey of a Group of Sikh Soldiers	655
RADHAKRISHNA RAO, M. V. Studies on Vitamin-A Deficiency. Part III. Lesions of the Peripheral Nervous System	661
MAJUMDAR, B. N., and DE, N. K. The Oxalic-Acid Content of some Indian Foodstuffs	671
RANGANATHAN, S. The 'Available' Iron in some common Indian Foodstuffs, determined by the <i>a, a'</i> -dipyridine Method	677
SUNDARARAJAN, A. R. Phytin-phosphorus Content of Indian Foodstuffs ..	685
PAL, R. K., and NARINDRA SINGH. The Effect on Rats of Supplementing a South Indian Diet with Calcium and Phosphorus	693
MAHAL, H. S. Studies on Blood Choline Esterase	703
DEY, B. B., LAKSHMIKANTHAM, P., DAVID, J. C., and KRISHNASWAMI, R. Preparation, Properties, and Pharmacological Action of p-Aminobenzoyl-aminomethyl-1-hydrocotarnine	713
SOKHEY, S. S., GOKHALE, S. K., MALANDKAR, M. A., and BILLIMORIA, H. S. Red Cells, Haemoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part II. Normal Indian Women: A Study based on the Examination of 101 Women	723
SANKARAN, G., and RAJAGOPAL, K. Haematological Investigations in South India. Part I. The Estimation of Haemoglobin	741
SANKARAN, G., and RAJAGOPAL, K. Haematological Investigations in South India. Part II. The Effect of the Administration of Iron on the Haemoglobin Level in Indian Girls and Young Women	753
SHORTT, H. E., PANDIT, S. R., MENON, K. P., and SWAMINATH, C. S. The Absence of Effective Immunity after Cure of Protozoal Infections ..	763
GHOSH, B. N., and DE, S. S. Investigation on the Isolation of the Neurotoxin and Haemolysin of Cobra (<i>Naja naja</i>) Venom	779
SIXTON, J. A. The Successful Transmission of Cutaneous Leishmaniasis by Inoculation to Man from a Natural Lesion occurring on a Dog in India ..	787
CHAKRAVARTI, D. N., and TYAGI, N. Studies in 'Effects of Heat'. Part I. Biochemical and Physical Changes in Ten Cases suffering from 'Effects of Heat'	791
NOTICE <i>re.</i> the League of Nations' Fifth International Course in Malariology ..	829
NOTICE <i>re.</i> University Readership in Medical Parasitology at the London School of Hygiene and Tropical Medicine	833

No. 4 (April 1938).

DHARMENDRA and JOHN LOWE. Attempts to Cultivate <i>M. lepræ muris</i> ..	835
GOYAL, R. K. A Serological Study of the Actinomyces	843

	PAGE
SWAMINATHAN, M. The Relative Amounts of the Protein and Non-Protein Nitrogenous Constituents occurring in Foodstuffs and their Significance in the Determination of the Digestibility Co-efficient of Proteins ..	847
DE, N. K., and MAJUMDAR, B. N. Further Studies on Factors affecting the Vitamin-A Activity of Animal and Vegetable Products ..	857
NARASIMHAMURTHY, G. The Nitrogen Complex of Indian Foodstuffs. Condiments : Part II. Chillies (<i>Capsicum annum</i>) and Coriander Seeds (<i>Coriandrum sativum</i>)	863
GIRI, K. V. The Availability of Phosphorus from Indian Foodstuffs ..	869
WILSON, H. E. C.; and ROY, G. K. The Flavine and Vitamin-B ₆ (Anti-dermatitis) Content of Indian Foodstuffs	879
GOSWAMI, H., and BASU, U. P. 'Available' Iron in Indian Foodstuffs ..	893
WILSON, H. E. C., and ROY, N. C. Observations on the Basal Metabolism of Indian Boys in Calcutta	901
KAMALA BHAGVAT. Enzyme Method for the Estimation of Adrenaline in Suprarenal Glands	911
DE, P., and CHATTERJEE, C. C. The Rôle of Formalin in the Estimation of Nitrogen in Body Fluids	917
DE, P., and BHATTACHARYYA, S. A Comparative Study of Glycolysis in Normal and Diabetic Blood	923
DE, P., and BHATTACHARYYA, S. Glycolysis in Drawn Blood in Twenty-four Hours	933
NANDI, B. K., and DIKSHIT, B. B. A New Colorimetric Test for Plasmogluine	937
SANKARAN, G., and RADHAKRISHNA RAO, M. V. Hæmatological Investigations in South India. Part III. The Mean Red Cell Diameter ..	951
RADHAKRISHNA RAO, M. V. Hæmatological Investigations in South India. Part IV. Hæmoglobin in Normal Pregnancy	957
SANKARAN, G., and RAJAGOPAL, K. Hæmatological Investigations in South India. Part V. The Effect of Altitude on Hæmoglobin Content ..	971
INDEX OF AUTHORS	979
INDEX OF SUBJECTS	987

A DIET SURVEY REPEATED AT ANOTHER SEASON.

BY

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AND

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[Received for publication, March 31, 1937.]

WE have recently reported the results of diet surveys carried out in various South Indian villages (Aykroyd and Krishnan, 1937). This investigation included the study of 32 families in the neighbourhood of Mayanur, Trichinopoly district, during July and August 1936. It was pointed out that a single diet survey covering a period of 20 days might give a false impression of dietary habits throughout the year, because seasonal variation might be considerable. In February 1937 an opportunity arose of organizing a further survey in the Mayanur district, and we felt that the repetition of a survey at a different season would be as valuable as an entirely new investigation in another district.

The inquiry reported here includes 25 families, 21 of which were studied 6 months previously. As before, they represented a cross-section of a village community. A number of families were unwilling to undergo a second inquisition. It did not prove possible to carry out a 20 days survey in all cases, and the actual periods of inquiry were as follows:—

20 days	11 families
19 "	4 "
15 "	3 "
13 "	1 family
9 "	6 families

In the case of the families studied for only 9 days, the investigation had to be discontinued owing to the intervention of a local festival lasting for a week.

The methods of collecting and working up the data were the same as those described in the previous paper. Workers attached to the Servants of India Society Rural Centre, Mayanur, who participated in the previous inquiry, visited homes twice daily to weigh out the food and make records. Intake of calories, proximate principles, and minerals have been worked out as before, the International scale of family co-efficients (League of Nations Health Organization, 1932)

A Diet Survey Repeated at Another Season.

TABLE.

Intake of calories, proximate principles, calcium, phosphorus, and iron per consumption unit per day.

Family.	Number of persons.	Number of consumption units.	Protein.	Fat.	Carbo-hydrate.	Calories.	Percentage of total calories derived from cereals.	Calorie intake in previous investigation.	Calcium (g.).	Phosphorus (g.).	Iron (mg.).
1	3	2.0	104.4	53.9	750.1	3,917	71	3,999	0.56	2.87	61.2
2	6	4.8	97.1	46.1	672.8	3,509	81	2,761	0.47	2.82	58.5
3	6	3.4	82.7	23.1	694.9	3,333	93	2,941	0.34	2.82	47.7
4	3	2.6	69.7	23.8	706.0	3,332	86	3,225	0.31	2.59	34.1
5	2	1.8	71.6	18.7	709.5	3,297	89	2,206	0.29	2.63	34.4
6	4	2.8	68.2	14.4	662.3	3,065	91	2,817	0.25	2.46	35.0
7	3	2.1	73.2	23.2	593.3	2,887	93	..	0.28	2.19	41.7
8	12	9.2	79.2	25.3	560.8	2,796	84	2,464	0.41	2.28	49.8
9	3	2.5	91.4	32.7	522.9	2,760	93	1,403	0.45	2.59	61.6
10	7	5.5	58.8	14.3	577.0	2,686	92	2,236	0.20	2.11	25.8
11	11	8.4	61.8	17.8	522.9	2,509	93	2,300	0.66	1.99	38.0

Geo	12	5	3.2	51.5	9.0	549.0	2,489	94	2,300	0.23	1.28	22.1
	13	4	2.8	50.4	90.7	330.9	2,390	44	2,453	0.83	1.14	10.7
	14	3	2.0	52.1	59.1	412.9	2,384	61	2,394	0.34	0.02	20.0
	15	2	1.6	54.6	24.3	481.2	2,370	79	..	0.35	1.85	24.8
	16	4	3.0	08.9	32.7	440.0	2,350	93	2,576	0.23	1.82	41.6
	17	5	3.5	57.4	19.6	479.6	2,331	89	2,391	0.32	1.81	33.2
	18	4	3.0	48.5	52.7	372.7	2,168	53	..	0.62	0.94	15.2
	19	6	4.8	56.5	21.7	413.6	2,082	76	2,721	0.45	1.66	29.0
	20	9	5.5	37.7	12.1	433.1	2,009	89	1,818	0.15	1.51	18.6
	21	3	2.5	60.0	32.5	353.7	1,950	66	..	0.49	1.07	25.1
	22	8	3.5	41.4	12.9	404.1	1,906	85	2,237	0.18	1.42	22.2
	23	5	4.5	41.4	7.2	402.4	1,817	90	1,591	0.19	1.53	19.6
	24	7	5.6	52.2	13.5	363.1	1,791	94	2,776	0.19	1.62	29.0
	25	4	2.8	37.9	15.6	317.4	1,570	82	1,215	0.18	1.24	19.4
Total ..		129	Mean:—3.76	63.1	27.9	500.6	2,550	82	2,420	0.36	1.91	33.2

being used to calculate the number of consumption units in each family (its man value). Analyses of Indian foodstuffs have been carried out in the Laboratories (Ranganathan *et al.*, 1937).

ANALYSES OF DIETS.

Intake of calories, proximate principles and minerals, and the percentage of total calories derived from cereals, are shown in the Table. Families are arranged in descending order of calorie intake. The calorie intake recorded in the previous investigation is given for purposes of comparison.

Mean calorie intake in the whole group (2,550) was higher than that calculated in the previous study for 29 families in the same district (2,400). Taking only the 21 families investigated twice, the means in both investigations are 2,420 and 2,588 respectively. Calorie intake was definitely higher in the majority of cases, only a small percentage showing a decrease. The general increase was largely due to a greater consumption of cereals, which probably resulted from the fact that February is a harvest season.

In spite of the higher calorie intake, mean protein intake was the same as in the earlier study (63.0 g. as compared with 62.7 g.). This is due to the fact that more rice and less millet were consumed. Average fat intake was also the same in both investigations (26.9 g. and 27.8 g.). The mean of the whole group of 29 families studied in 1936 is taken here as the basis of comparison.

Mean intake of calcium, phosphorus, and iron per consumption unit daily in the two inquiries compared as follows:—

Year.	Calcium (g.).	Phosphorus (g.).	Iron (mg.).
1936 ..	0.31	1.51	32.8
1937 ..	0.36	1.90	33.1

Calcium intake was again below textbook requirements. Recent animal experiments have suggested that one of the most important defects of the South Indian diet is its deficiency of calcium. Phosphorus intake was higher because of the larger proportion of home-pounded rice in the diet. Chemical investigations have shown that washing and cooking deprive rice of about 70 per cent of its phosphorus. Even if this loss is allowed for, average phosphorus intake works out at 1.4 g. per consumption unit daily.

Vitamin A was for practical purposes absent from the diet of 15 families. In the remaining families average intake was 120 γ per day (312 International units). Mean carotene intake was equivalent to about 750 International vitamin-A units, a figure corresponding to that obtained in the first study.

Milk.—The intake of milk products was not noticeably larger than that recorded in the previous investigation. Thirteen out of the 25 families consumed

no milk products at all. Five more consumed less than 1·0 oz. per consumption unit daily of milk or curds. Intake in the remaining families was as follows :—

Family.	Product.			Quantity per consumption unit per day (oz.).
2	Butter-milk	5·0
13	Whole milk	12·7
	Curds	3·9
14	Curds	0·7
	Ghee	0·9
15	Whole milk	1·6
	Curds	1·6
18	Whole milk	7·8
	Curds	4·0
	Ghee	0·6
19	Whole milk	4·6
	Goat's milk	0·6
21	Goat's milk	2·4
	Curds (from cow's milk)	2·6

The families consuming several ounces of milk products daily were in general more prosperous than the remainder. Family 18 was that of an agricultural instructor attached to the Rural Centre, 13 a Brahmin family in relatively comfortable circumstances.

Animal foods other than milk.—Seven families ate mutton, fish, or chicken in very small quantities, the highest intake being 1·0 oz. per consumption unit daily. One family had a few eggs. The remainder did not consume flesh foods or eggs.

Pulses.—All the families consumed pulses, the lowest and highest intakes being 0·3 oz. and 3·0 oz. respectively. Mean intake was about 1·3 oz., corresponding to that recorded in the previous inquiry (1·3 oz.).

Vegetables.—Consumption of green leafy vegetables was even lower than in the earlier investigation. They were included in small quantities in the diet of only 4 families. This confirms our general experience that green leafy vegetables form a very unimportant part of the diet of South Indian peasants throughout the year.

Non-leafy vegetables included brinjals, sweet potatoes, gourds, cluster beans, drumstick, and a number of other varieties. Intake of such vegetables was

definitely higher than in July and August 1936. Average daily intake was about 3.0 oz. per consumption unit, as compared with less than 1.5 oz. in the previous investigation.

Fruits.—Fresh fruit was absent from the diet of 16 families. The remainder consumed plantains in small quantities. One family had a few limes, another a few tomatoes. Some fresh fruits may be plucked and consumed out of doors particularly by children.

As before, it was observed that even the poorest families consumed a fair quantity of dried tamarind daily. Mean intake was 0.63 oz. per consumption unit per day, as compared with 0.48 oz. in the previous inquiry.

Vegetable oils.—All the families except one consumed gingelly oil or ground-nut oil, some in very small quantities. Coco-nut was included in the diet of 15 families. As in the earlier investigation, average intake of vegetable oil was in the neighbourhood of 0.5 oz. per consumption unit daily.

DISCUSSION.

Diets consumed were in general similar during the two periods of inquiry in July and August 1936, and February 1937, respectively. The correspondence confirms the substantial accuracy of the basic data. Calorie intake was appreciably higher in the second investigation and rather more non-leafy vegetables were consumed. In general, however, diets at both seasons show the same preponderance of rice and millet and relative lack of 'protective' foods such as milk, green leafy vegetables, and fruits. It may probably be concluded that the quality of the diet consumed by poor South Indian peasants does not vary much with season.

SUMMARY.

A diet survey was carried out on a group of South Indian peasant families whose food consumption had been investigated 6 months previously. Intake of calories and consumption of vegetables were higher in the second investigation, but in general a similar picture of dietary habits was obtained at both seasons.

ACKNOWLEDGMENTS.

We must again thank Mr. K. G. Sivaswamy and his colleagues of the Servindia Rural Centre, Mayanur, who visited families and collected food consumption data, for their ungrudging, laborious, and conscientious assistance.

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 RANGANATHAN, SUNDARARAJAN and SWAMINATHAN (1937). *Ind. Jour. Med. Res.*, **24**, p. 689.

RED-PALM OIL IN THE TREATMENT OF HUMAN KERATOMALACIA.

BY

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[Received for publication, March 31, 1937.]

RED-PALM OIL is derived from the fruit of the West African palm *Elæis guineensis*. This palm has been imported into Malaya and Burma; in the former country it appears to be extensively grown, but not as yet in the latter. It has not yet been cultivated in India.

Drummond and Coward (1920) were the first to observe that red-palm oil possesses vitamin-A activity as shown by biological tests. They observed that its pigment is very largely carotene. Further work on the vitamin-A activity of red-palm oil was carried out by Drummond, Ahmad and Morton (1930), and Ahmad (1931). The former workers reported that samples of red-palm oil had an activity equal to that of cod-liver oil of more than average potency; young rats depleted of vitamin A resumed growth when 2 mg. of palm oil were added to the diet. Ahmad showed that 5 mg. daily are sufficient to cover the vitamin-A requirements of the rat. Biological tests of palm oil were also carried out by Rosedale and Oliveiro (1934). Rosedale (1935) reported that red-palm oil contained 1,200 yellow units of carotene per g.; of a series of Malayan foodstuffs tested it was by far the richest in carotene. In the Coonoor Laboratories it has recently been found that samples of Malayan and Burmese red-palm oil contain about 500 γ of carotene per g., having a carotene content equal to the vitamin-A content of a good sample of cod-liver oil.

There thus appears to be great possibilities in red-palm oil as a cheap source of vitamin-A activity in the Tropics and the East, both for use as a food and as a medicine for the treatment of vitamin-A deficiency. It is, however, devoid of vitamin A (*strictu sensu*) and hitherto it has not been conclusively shown that

carotene can fully replace vitamin A in the diet of human beings. Nor has its effect in cases of keratomalacia been observed; it seemed possible that, in such cases, efficient transformation of carotene into vitamin A might not take place. It was therefore felt desirable to carry out clinical tests of red-palm oil before taking steps to increase and popularize its production and consumption.

LABORATORY AND CLINICAL OBSERVATIONS.

A large sample of red-palm oil was obtained from Malaya. Its carotene content was spectrographically assayed by De's (1937a) method and found to be about 500 γ per gramme. To test the biological activity of the oil, experiments were carried out on rabbits. Young rabbits, fed on the following diet, develop in three to four months an eye condition closely resembling keratomalacia in human beings:—

	Parts.
Crushed oats	68.5
Rice bran	20.0
Calcium carbonate	1.5
White potatoes (to supply vitamin C)	10.0

The characteristic eye lesions were cured in a few days by 0.5 g. to 2.0 g. of red-palm oil. Four curative tests of this nature were carried out.

Clinical.—The following report on the clinical experiments (sent to the Nutrition Research Laboratories by R. E. W.) provides a sufficient summary of results:—

“In the first instance, we sought and obtained the help of Colonel E. S. Goss and Mr. McCulloch of the Medical Stores Department with regard to the best method of dispensing the oil. After a number of experiments they gave us two emulsions A and B to choose from, and we adopted A since B was too thick for convenient and economic use when issued to patients, and moreover the oil tended to separate out. The emulsion A was as follows:—

Red-palm oil	80 minims	(4.7 c.c.).
Gum ghatti (Indicum)	25 grains	(1.69 g.).
Gum tragacanth	10 „	(0.64 g.).
Cinnamon oil	0.12 minim	(0.007 c.c.).
Peppermint oil	0.4 „	(0.023 c.c.).
Chloroform	1.0 „	(0.059 c.c.).
Glucose liquid	150 grains	(9.6 g.).
Water to	1 oz.	(28.35 c.c.).

“[Place the palm oil, gum ghatti, and gum tragacanth in a dry mortar. Grind to smooth emulsion. Add the glucose and 2 drachms of water and mix to a homogeneous emulsion. Take one minim of cinnamon oil and 2 minims of peppermint oil and make up to 8 minims with rectified spirit. Take one minim of chloroform and mix well.]

“We adopted the dose previously agreed upon, namely, an amount equal to 10 minims (0.59 c.c.) of the original oil as an average dose for children between 5 and 10, a suitable allowance being made for younger and older individuals. This was

given twice daily. Clinical inquiries of this nature involve difficulties and fallacies, one of which is the possibility that a beneficial effect on cases of deficiency may result from simply admitting the patients and placing them on hospital diet. In three instances, however, we were enabled to treat and follow up cases suitable for observation which remained living under the identical domestic conditions in which they developed the syndrome. The only change in their daily routine was the addition of red-palm oil emulsion to their diet. In these the improvement was so definite that we concluded that, in so far as the strictly limited observation goes, the red-palm oil *per se* acted in a curative capacity supplying the necessary factor or factors in the same way as we had found cod-liver oil act previously. Most of the cases of keratomalacia treated in the hospital are infants and young children. Cessation of deterioration may be observed within a week and a definite improvement within a fortnight from the commencement of treatment with the red-palm oil.

“With regard to the routine treatment of cases admitted to the hospital, we tried as far as possible to compare the results of treatment with red-palm oil with those of cod-liver oil. Dr. Narayanaswami Pilla assisted by Dr. Muhamad Auzum Sahib was in immediate charge of the cases and Rao Bahadur Dr. K. Koman Nayar and I acted as assessors inspecting the cases at intervals. We came to the following conclusions:—

(1) That the red-palm oil in the amounts indicated above gave results comparable to cod-liver oil.

Note.—The cod-liver oil emulsion which we ordinarily use contains 60 minims (3.55 c.c.) of cod-liver oil to the ounce and the average dose for children is 15 minims (0.88 c.c.) of the original oil. This is slightly modified for babies and adults.

(2) That in case of juvenile or infantile diarrhoea, it was better borne than cod-liver oil which tended to aggravate such symptoms.

(3) That whereas in this latter type of cases, cod-liver oil supplied on a binder was an effective method of treatment, we had not sufficient evidence to say the same of red-palm oil. (This is mainly because, in babies who were very ill or whose eyes were in a very critical condition, we did not feel justified in withholding a known remedial agent.)

(4) That red-palm oil emulsion is more palatable than cod-liver oil emulsion.

“In so far as clinical investigation of this nature goes, we feel justified in making the statement that red-palm oil—as suggested by you—forms an effective substitute for cod-liver oil in the routine treatment of the average keratomalacia case.

“We reserve judgment in the case of babies *in extremis*. We propose to continue these clinical investigations during 1937, and, except in the latter type of cases, propose the substitution of red-palm oil emulsion for cod-liver oil in order to get an impression as to the result of, say, a year's experience by comparison with previous years, when only cod-liver oil was used”.

The quantities of red-palm oil and cod-liver oil given daily were approximately 1.1 grammes and 1.5 grammes respectively. This would supply over 500 International units per day.

THE COST OF RED-PALM OIL.

Red-palm oil is obviously a valuable potential source of an essential vitamin. It is, however, difficult to make a satisfactory estimate of its probable cost should it come to be used on a wider scale as a medicine or a dietary ingredient. Red-palm oil, shipped in bulk from Singapore, costs at present 183 Straits dollars per ton. This would work out at approximately 2 annas per pound. Cod-liver oil in India costs from 10 annas to 1 rupee per pound. The cost, when purchased by the public, includes a duty of 20 per cent *ad valorem* when imported from countries within the British Empire and 30 per cent when imported from other countries.

The average vitamin-A content of seven samples of cod-liver oil tested in the Laboratories was about 300 γ of vitamin A per gramme, while the carotene content of 3 samples of red-palm oil averaged about 500 γ per gramme. Most of the cod-liver oil samples were obtained by local purchase. One γ of carotene and 1 γ of vitamin A, as estimated by De's method, are roughly equivalent to 1.0 and 2.6 International units respectively. On the basis of these figures, 1 lb. of red-palm oil will contain roughly 0.6 of the vitamin-A activity of a similar quantity of cod-liver oil. If the cost of palm oil and cod-liver oil is taken as about 2.5 annas and 12 annas per lb. respectively, the amount of vitamin-A activity purchasable for a given sum in the form of red-palm oil is roughly three times greater than that purchasable in the form of cod-liver oil.

Clearly, then, red-palm oil merits attention on the part of nutrition workers in the Tropics and the East. In Malaya it has been successfully used in clinical work (Rosedale, personal communication). The fact that, unlike cod-liver oil, it contains very little or no vitamin D (Dann, 1932) is not necessarily a drawback to its use in countries where vitamin D is supplied by abundant strong sunlight and rickets is rare. In addition to being used in hospitals and dispensaries, it might be consumed as an ordinary ingredient in the diet, mixed with other vegetable fats devoid of carotene. According to Dann (*loc. cit.*) 'it is used by the natives of West Africa as a culinary oil in much the same way that butter and olive oil are employed in European countries'. Experiments on the palatability and nutritive value of mixtures of red-palm oil and other fats and oils are reported in an accompanying paper in this issue (De, 1937b).

The question of introducing the palm *Elæis guineensis* into South India is worth attention on the part of agricultural authorities. The climate of South India would probably be suitable for its cultivation.

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THE POSSIBLE USE OF RED-PALM OIL IN SUPPLEMENTING THE VITAMIN-A ACTIVITY OF COMMON VEGETABLE OILS.

BY

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It has been proved beyond doubt that red-palm oil is a very potent source of vitamin-A activity (Drummond and Coward, 1920 ; Drummond, Ahmad and Morton, 1930 ; Ahmad, 1931 ; Rosedale, 1935 ; De, 1936, 1937). The pigment of the oil is almost entirely carotene which alone is responsible for its vitamin-A activity. It possesses an activity of the same order as that of cod-liver oil, or some ten times greater than that of butter. Red-palm oil appears to be the only oil of vegetable origin which possesses vitamin-A activity to a notable degree, and further, it is so highly potent that the question of its production and use deserves special attention. It is of obvious importance to discover whether red-palm oil can be used medicinally, in hospitals . . . as a substitute for the more expensive cod-liver oil ; and whether it can suitably be used, in India and elsewhere, as an ingredient in diets which are relatively deficient in vitamin A and carotene. The first question has been dealt with by Aykroyd and Wright (1937) in this issue ; the present investigation deals with the second problem.

The palm *Elais guineensis*, from which red-palm oil is derived, is cultivated chiefly in tropical Africa. According to Dann (1932), natives of West Africa are accustomed to use red-palm oil in the same way as butter and olive oil are employed in European countries.

A sample of oil obtained from Malaya for experiment possessed a smell and taste which, while not seriously unpleasant, could scarcely be called appetizing. The author gave the oil a trial as cooking oil in his own household, and small quantities were distributed to numerous friends with a request to do the same. The general verdict was unfavourable ; red-palm oil as such cannot be recommended as a palatable food.

Next attempts were made to discover whether red-palm oil could be more agreeably consumed when mixed with common vegetable oils, such as coco-nut

12 Red-Palm Oil Supplementing Vitamin-A Activity of Vegetable Oils.

oil, mustard oil, gingelly oil, olive oil, and Cocogem. When the proportion of red-palm oil was small (6 to 12 per cent) these mixtures were found to be perfectly palatable; no objectionable taste or smell was imparted to foods cooked in them. The 'palm oil' taste appeared to be masked by the taste of the oil with which it was mixed, and the resulting preparation differed from the original oil only in having a reddish colour.

Small quantities of red-palm oil were mixed with butter and a hardened fat (margarine). These mixtures were palatable when used in cooking but disagreeable in the raw state, e.g., when spread on bread.

EXPERIMENTAL.

The various mixed oils, including 6 to 12 per cent palm oil, contained about 30γ to 70γ of carotene per gramme, their vitamin-A potency being 1 to 3 times that of good butter. An investigation was undertaken to discover the effect of cooking on the carotene content of such oils. The most drastic heat-treatment of cooking oils occurs in frying, so carotene losses during frying were systematically studied.

Potatoes (containing traces of carotene) were cut into thin round chips; these were salted and fried in the various oil mixtures described in the Table. The heated oils and the fried chips were assayed for carotene. The data are given in the Table:—

TABLE.

Effect of heat on the carotene content of various mixtures of palm oil and other oils.

Experiment.	Nature of the mixture and heat treatment.	Carotene content of the test material γ (0.001 mg.) per gramme.
I.	Red-palm oil (original)	520.0
	„ „ heated on water-bath for 5 hours (maximum temperature about 90°C.).	115.2
	„ „ heated over direct flame for ½ hour (maximum temperature above 250°C.).	Nil.
	„ „ after being used for frying potato chips (single operation).	216.0
	„ „ after the second frying	144.0
	„ „ after the third frying	63.0
II.	Red-palm oil and coco-nut oil (1 : 10 approx.)	51.0
	„ „ after the first frying	34.0
	„ „ after the second frying	28.0
	„ „ after the third frying	24.5
	„ „ after the fourth frying	23.0
	„ „ after the fifth frying	20.4
	Potato chips fried in original mixed oil	8.0
	„ „ in the fifth frying	5.0

TABLE—concl'd.

Experiment.	Nature of the mixture and heat treatment.			Carotene content of the test material γ (0.001 mg.) per gramme.
III.	Red-palm oil and Cocogem (1 : 8 approx.)	72.0
	" " after the first frying	54.0
	" " after the fifth frying	30.0
	Potato chips fried in the second frying	9.0
IV.	Red-palm oil and mustard oil (1 : 16 approx.)	36.0
	" " after the third frying	21.6
	Potato chips fried in the third frying	6.0
V.	Red-palm oil and gingelly oil (1 : 16 approx.)	35.0
	" " after the first frying	28.0
	" " after the second frying	25.0
	" " after the third frying	19.6
	" " after the fourth frying	14.4
	Potato chips fried in the original mixed oil	7.0
VI.	Red-palm oil and ground-nut oil (1 : 10 approx.)	54.0
	" " after the third frying	27.0
	Potato chips fried in the original mixed oil	10.0
VII.	Red-palm oil and olive oil (1 : 7 approx.)	74.0
	" " after the third frying	30.0
	Potato chips fried in the third frying	9.0

The frying of potato chips was carried out in a wide-mouthed silica basin (capacity 1 litre). One hundred to two hundred c.c. of the oil or oil mixture was poured into it, and the oil heated, over a small Bunsen flame, to about 130°C. to 140°C. the fluid being occasionally stirred by a spoon; when absence of frothing indicated that the oil was ready for frying, the chips were placed in the basin. On addition of the chips the temperature dropped down to about 100°C. and then rose slowly. So long as the chips contained moisture, the temperature did not rise much; afterwards, however, it rose rapidly. The range of temperature during the whole operation of frying was between 100°C. and 140°C. The potato chips were fried for 15 to 25 minutes until the chips became stiff. In each operation the number of chips fried was suitably adjusted, in a culinary sense, to the size of the basin and the amount of oil used. Specimens of fried chips used for analysis were wiped with blotting-paper to free them from the adhering fat. The carotene content of the various heat treated oils and the fried chips was estimated colorimetrically by comparison with a standard solution whose carotene content has been previously determined by a spectrographic method.

14 *Red-Palm Oil Supplementing Vitamin-A Activity of Vegetable Oils.*

It may be added that the chips fried in the various mixtures were found to be perfectly palatable by the author and others. The same could not be said of the chips fried in pure palm oil.

The experiments described were devised to investigate a practical point, and it was felt that there would be no advantage in carrying this out on a strictly quantitative basis, e.g., with weighed quantities of oil and potato, and with the frying process exactly standardized as regards time and temperature.

DISCUSSION.

It is apparent from the Table that carotene is liable to destruction by heat. The loss depends on the severity of the heat, time of heating, etc. During each frying operation, the oils lost some carotene. But even after several fryings, the vitamin-A activity of the oil mixtures was not less than half that of *uncooked* butter or ghee. Butter and ghee used for frying will likewise lose vitamin A, and after a number of fryings will be considerably less potent than the oil mixtures described.

If mixtures of red-palm oil and other oils can retain carotene to this extent after repeated frying, it may be assumed that the application of less drastic cooking measures to such mixtures will not involve serious impairment of their vitamin-A activity.

The fried chips contained 5γ to 10γ of carotene per gramme. Suppose 15 to 20 grammes of free fat in the form of mixtures of red-palm oil and other oils in the proportion of 1 to 10 are included in the daily diet. Actual diet surveys have shown that Indian diets may contain about this quantity of free fat (Aykroyd and Krishnan, 1937). Even when losses due to cooking are allowed for to the fullest extent, 15 grammes of such mixtures might supply about 500γ of carotene. It has been observed (Aykroyd and Krishnan, *loc. cit.*) that the average carotene content of the diet of groups of Indian peasant families is in the neighbourhood of 700γ per consumption unit daily. An addition of about 500γ would be of the utmost value.

One hundred grammes of food which has been fried in the mixtures would yield about 500γ to 1,000γ of carotene.

There seems no reason why the common vegetable oils or fats in the market should not be enriched with palm oil in the manner described. The price of red-palm oil in bulk appears to be of the same order as that of vegetable fats in common use in India. Vitamin-A deficiency is so common in India, and rich sources of vitamin A and carotene so rare and expensive, that no feasible method of making good the deficiency should be neglected. The production of such mixtures in bulk may be suggested to commercial organizations.

SUMMARY.

Mixtures of common vegetable oils and red-palm oil were prepared. These mixtures possess high vitamin-A activity and are sufficiently palatable for use in cooking. While loss of carotene occurs in cooking, enough will remain in the oil

and the materials cooked in it to be of value in making good one of the most serious deficiencies in typical Indian diets.

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THE ADSORPTION OF VITAMIN A AND CAROTENE AND THE ISOLATION OF VITAMIN A FROM ASSOCIATED PIGMENTS.

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It has long been known that charcoal is a very powerful adsorbent for various pigments. This property has been applied for removing carotene from crude vitamin-A solutions by several workers (Gillam *et al.*, 1933, etc.), though not with strictly quantitative purposes. Stephenson, as early as 1920, noted that charcoal could remove carotene from butter without affecting its vitamin-A activity as judged by animal experiments. Karrer, Walker, Schopf and Morf (1933) have separated a vitamin-A concentrate into two fractions by fractional adsorption on lime; the existence of various kinds of lipochrome pigments, including different forms of carotene and xanthophylls, etc., was clearly revealed by means of chromatography on lime, aluminium oxide, calcium carbonate, and other adsorbents. Bowden and Bastow (1934) and Homes *et al.* (1933) have done a considerable amount of work on the adsorption of vitamin A and carotene. Their work was undertaken mainly to discover a means of preparing vitamin-A concentrates. They reported that carotene is much more easily adsorbed than vitamin A and that the degree of adsorption depends on the nature of the solvent, adsorption being more rapid from a non-polar solvent such as cyclohexane than from a polar solvent, e.g., chloroform. The adsorbed factor could be recovered from the adsorbents by a suitable solvent apparently unchanged.

Apart from the experimental work mentioned above, no thorough examination has yet been carried out to see if charcoal adsorption can be used for a quantitative separation of vitamin A and carotene, a problem of great technical importance in vitamin-A assay work. The present paper records an investigation of this problem.

EXPERIMENTAL.

Experiments were conducted (1) to study the relative adsorbability of vitamin A and carotene from different solvents and by different adsorbents; (2) to discover whether vitamin A can be freed from carotene (and other similar associated pigments) by use of adsorption methods.

Different stock solutions of vitamin A and carotene (50 γ /c.c.) were first prepared by dissolving samples of cod-liver oil and red-palm oil of known potency in the various solvents. Several 10-c.c. samples of a given solution were transferred to conical flasks of 50-c.c. capacity and each treated with one gramme of charcoal (Norit, Super, Baird and Tatlock, London). These were immediately shaken under identical conditions (in an electrically-driven shaking machine, 100 shakings per minute) for different periods of time and filtered immediately. The various filtrates were tested for vitamin A or carotene. From these observations, the time required for complete removal of the factor concerned was easily determined. Similar experiments were conducted with each of the stock solutions in the various solvents. The absence of vitamin A and carotene was determined by a negative antimony trichloride test and by the disappearance of all yellow colour respectively. The results are set out in Table I:—

TABLE I.

Relative adsorbability of vitamin A and carotene from various solvents.

Solvent.	Approximate minimum time required for complete removal of carotene (contained in red-palm oil). Original strength: 50 γ of carotene per c.c. (Average of 3 observations).	Time required for complete removal of vitamin A (contained in cod-liver oil). Original strength: 50 γ of vitamin A per c.c.
Ether	2½ minutes	Adsorption of vitamin A on all the agents was nil or negligible. Even 8 hours' shaking, and subsequent keeping for 24 hours, removed only a little vitamin A from an ether solution. When the vitamin-A solution (in petrol-ether) was prepared from halibut-liver oil the adsorption was, however, much quicker.
Petrol-ether (boiling point 60°C. to 80°C.).	2½ "	
Hexane	2½ "	
Carbondisulphide ..	4 "	
Benzene	6 "	
Chloroform ..	1 hour not enough.	
Alcohol and acetone ..	The required amounts of red-palm oil and cod-liver oil did not go into solution.	

The original samples of red-palm oil and cod-liver oil contained, roughly, 500 γ of carotene and vitamin A per gramme respectively. The various solutions contained 100 mg. of free fat per c.c. The solutions of vitamin A prepared from halibut-liver oil contained only about 4 mg. of free fat per c.c.

From Table I it appears that in the presence of free fat (to the same extent in all cases) adsorption of vitamin A by charcoal was nil or negligible, while carotene was very strongly adsorbed under the same conditions. The degree of adsorption varied with the nature of the solvent, carotene being most easily removed from ether, petrol-ether, hexane, etc., while from chloroform adsorption was very slow. Most of the carotene or vitamin A adsorbed from ether, petrol-ether, hexane, benzene, or carbondisulphide solutions could be recovered by washing the adsorbate with chloroform. Throughout the rest of the investigation petrol-ether with a high boiling point (60°C. to 80°C.) was used as solvent, so that the concentration of the solutions would be least affected by spontaneous evaporation.

Solutions of vitamin A (50 γ per c.c.) were then prepared by dissolving halibut-liver oil (containing about 12,000 γ of vitamin A per gramme) in petrol-ether. In this case the amount of free fat in solution was very little (about 4.0 mg. per c.c.). Ten-c.c. samples of this solution were shaken under similar conditions, each with one gramme Norit charcoal. It was observed that about 30 minutes of shaking was necessary for complete removal of vitamin A. It follows from these observations that the rate of adsorption of vitamin A or carotene will depend on the amount of free fat present in solution.

Preliminary observations were then made with solutions of unsaponifiable fractions of halibut-liver oil and red-palm oil. It was noted that adsorption of either vitamin A or carotene in these cases was very much greater than that occurring in the presence of free fat although adsorption of vitamin A was comparatively slow. The increased adsorbability in the absence of free fat may be due to the less viscous nature of the fluid. Next an experiment was conducted to study how adsorption of carotene on different solid materials varies. Solutions of carotene (5.4 γ per c.c.) were made in petrol-ether from the unsaponifiable fractions of red-palm oil. Ten-c.c. samples were treated with one gramme of each of the various agents described in Table II, and shaken for 3 minutes in each case, under the same conditions as before. They were immediately filtered and the carotene content of the filtrate was determined colorimetrically by comparison with a pure carotene solution whose carotene content was previously determined by a spectrographic method (De, 1935, 1937). The results are set out in Table II.

From Table II it is seen that of all the agents used only charcoal adsorbed carotene rapidly, while the adsorbing effect of other agents was comparatively little. Adsorption of vitamin A by the various agents other than charcoal was also studied and found to be nil or negligible in the same conditions.

TABLE II.

*Relative adsorbability of carotene by the various solid materials
(petrol-ether solution of the unsaponifiable fraction
of red-palm oil).*

Adsorbing materials and quantities added to 10 c.c. of the solution.	Strength of the filtrate after 3 minutes' shaking. Original strength : 5.4γ of carotene per c.c. (Average of 2 observations).	Adsorption power (roughly in arbitrary figures).
	(in γ per c.c.).	
Charcoal (Norit) (100 mg. only)	0.0	10,000
Charcoal (ordinary) (100 mg. only).	0.5	9,000
MgO (1 gramme)	3.9	28
Fullers' earth (1 gramme)	4.2	22
Ba(OH) ₂	4.7	13
Ca(OH) ₂	4.7	13
Al ₂ O ₃	5.0	7
Silica	5.1	6
CaCO ₃	5.4	0
CaSO ₄	5.4	0
CaCl ₂	5.4	0

The study of how the adsorption of vitamin A and carotene varied with the quantity of charcoal used, time and rate of shaking, etc., was next undertaken. The data are set out in Tables III to VIII and expressed graphically in Figs. 1 to 5 :—

TABLE III.

*Adsorption of carotene by various amounts of charcoal
(unsaponifiable matter in petrol-ether).*

Amount of charcoal (Norit) (mg.).	Time required for complete removal of carotene. Original strength : 50γ of carotene per c.c. (Average of 3 observations).
200	30 seconds.
100	3 minutes.
70	7 "
50	40 "
10	1½ hours of shaking not enough for complete removal of carotene.

To 10-c.c. solutions (50 γ of carotene per c.c.) the stated amounts of charcoal were added and the solutions shaken under similar conditions until carotene was totally removed.

TABLE IV.

Adsorption of carotene in a given time by varying amounts of charcoal (unsaponifiable matter in petrol-ether).

Amount of charcoal (Norit) (mg.).	Carotene content of the filtrate. Original strength : 50 γ of carotene per c.c. (in γ per c.c.).
0	50.0
10	21.6
20	7.8
40	3.6
60	2.1
80	0.9
100	0.0

To 10-c.c. samples of the solution (50 γ of carotene per c.c.) the stated amounts of charcoal were added; each solution was shaken for 3 minutes under similar conditions and immediately filtered.

TABLE V.

Adsorption of carotene by a fixed amount of charcoal when shaken for a fixed time at various rates of shaking.

Number of shakings per minute.	Carotene content of the filtrate (unsaponifiable matter in petrol-ether). Original strength : 50 γ of carotene per c.c. (in γ per c.c.).
0	34.2
50	7.6
100	1.8
200	0.0

Original strength in each case was 50 γ of carotene per c.c. Ten-c.c. samples were treated each with 50 mg. of charcoal, shaken (with an electrically-driven

machine) for 3 minutes and immediately filtered. Mere keeping for 3 minutes without shaking caused the carotene value to fall from 50.0 γ to 31.0 γ per c.c.

TABLE VI.

*Adsorption of vitamin A by varying amounts of charcoal
(unsaponifiable matter in petrol-ether).*

Amount of charcoal (Norit) (mg.).	Time for complete removal of vitamin A. Original strength: 5.0 γ of vitamin A per c.c. (Average of 2 observations).
500	1 minute.
400	7 "
300	25 "
200	45 "
100	1½ hours were not enough.

From preliminary experiments it was noted that adsorption of vitamin A by charcoal was very much less than that of carotene. In this experiment 10-c.c. samples (extract of unsaponifiable fraction of halibut-liver oil, 5.0 γ of vitamin A per c.c. of the solution) were shaken under similar conditions at 100 shakings per minute with the stated amounts of charcoal until the filtrate was devoid of vitamin A as evidenced by a negative antimony trichloride reaction.

TABLE VII.

*Adsorption of vitamin A in a given time from a
given solution by varying amounts
of charcoal.*

Amount of charcoal (Norit) (mg.).	Vitamin-A content of the filtrate. Original strength: 50 γ of vitamin A per c.c. (in γ per c.c.).
0	5.00
10	3.20
50	2.50
100	1.60
200	0.62
300	0.31
400	0.12
500	0.00

Ten-c.c. fractions of the vitamin-A solution (petrol-ether extract of the unsaponifiable fraction of halibut-liver oil) were treated with the stated amounts of charcoal, shaken at 100 shakings per minute, for 3 minutes, and filtered immediately.

TABLE VIII.

Removal of carotene from a mixture of vitamin A (5 γ per c.c.) and carotene (50 γ per c.c.) in petrol-ether solution, in the presence and absence of free fat.

Nature of medium.	Amount of charcoal (Norit) (mg.).	Time of shaking (minutes).	Vitamin-A content of the filtrate. Original strength : 5.0 γ of vitamin A per c.c. (in γ per c.c.).	Carotene content of the filtrate. Original strength : 50.0 γ of carotene per c.c. (in γ per c.c.).
Free fat absent (halibut-liver oil and red-palm oil both saponified).	100	3	1.6	0.0
Free fat absent (halibut-liver oil and red-palm oil both saponified).	100	3	1.4	0.0
Very little fat present (halibut-liver oil and red-palm oil, latter saponified).	100	2	3.5	0.0
Very little fat present (halibut-liver oil and red-palm oil, latter saponified).	100	3	3.9	Trace.
Large quantities of fat present (cod-liver oil and red-palm oil).	500	3	5.0	Trace.
Large quantities of fat present (cod-liver oil and red-palm oil).	500	2	4.8	0.0
Large quantities of fat present (cod-liver oil and red-palm oil).	1,000	2	4.9	0.0
Large quantities of fat present (cod-liver oil and red-palm oil).	1,000	2	5.0	0.0
Large quantities of fat present (cod-liver oil and red-palm oil).	1,000	3	4.7	0.0

Ten-c.c. samples of the respective solutions in identical conical flasks were treated with the stated amounts of charcoal shaken by hand, till the solution was just decolorized, and then immediately filtered.

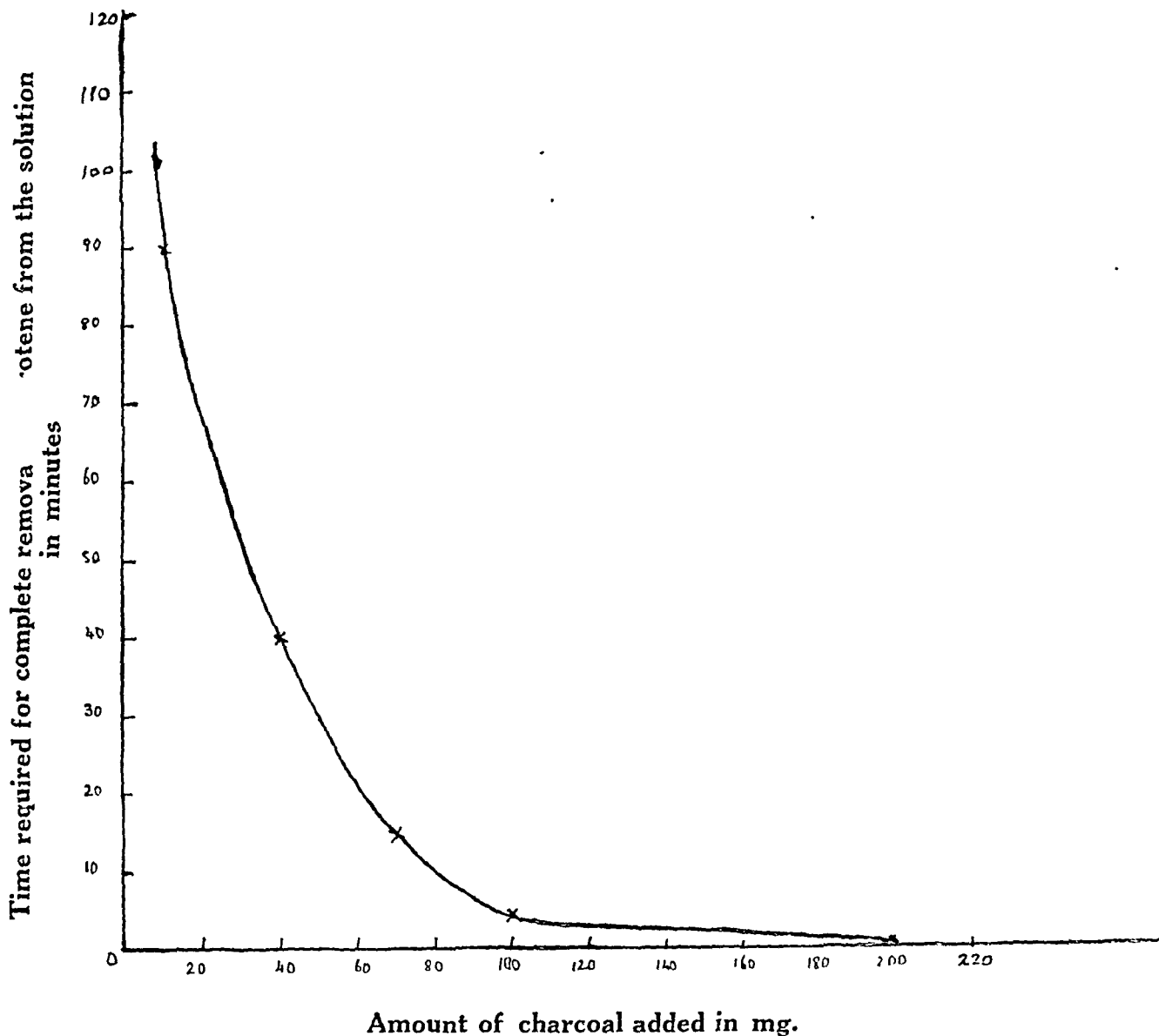


Fig. 1.—Showing how the time required for complete removal of carotene from solution varied according to the quantities of charcoal used. Ten-c.c. samples of the solution were treated with varying amounts of charcoal and shaken for different periods to determine the minimum time required for complete decolorization (cf. Table III).

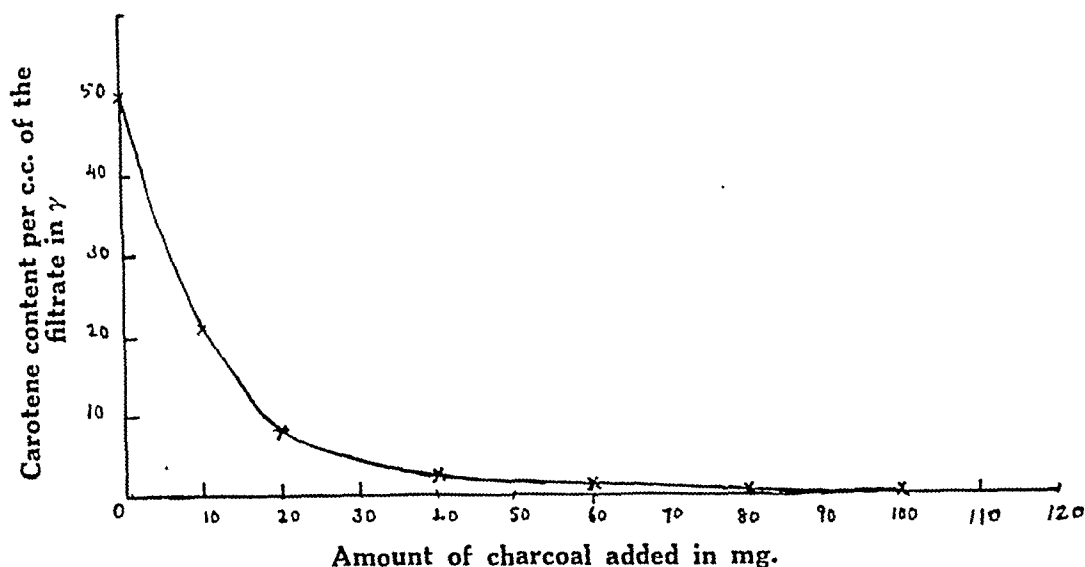


Fig. 2.—Showing how the adsorption of carotene varied according to the quantities of charcoal used. Ten-c.c. samples of the solution were treated with varying amounts of charcoal and each shaken for 3 minutes and filtered immediately (*cf.* Table IV).

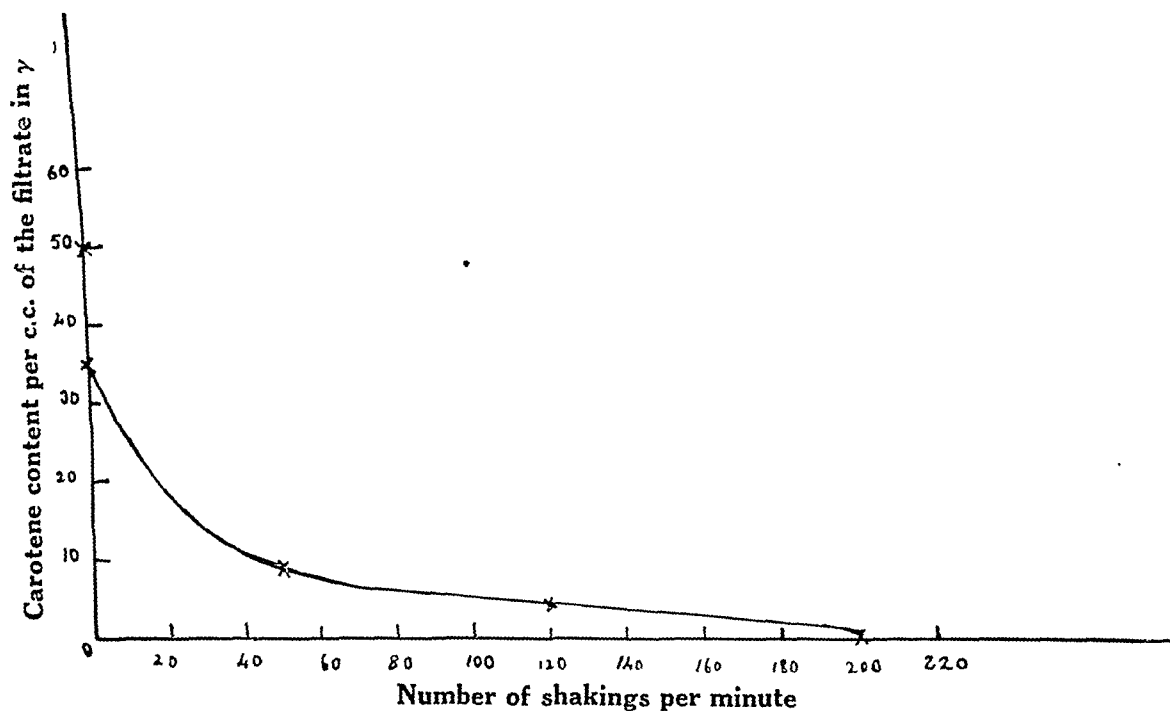


Fig. 3.—Showing how the adsorption of carotene varied according to the rate of shaking. Ten-c.c. samples of the solution were treated each with 50 mg. of charcoal, shaken at various rates for 3 minutes, and filtered immediately (*cf.* Table V).

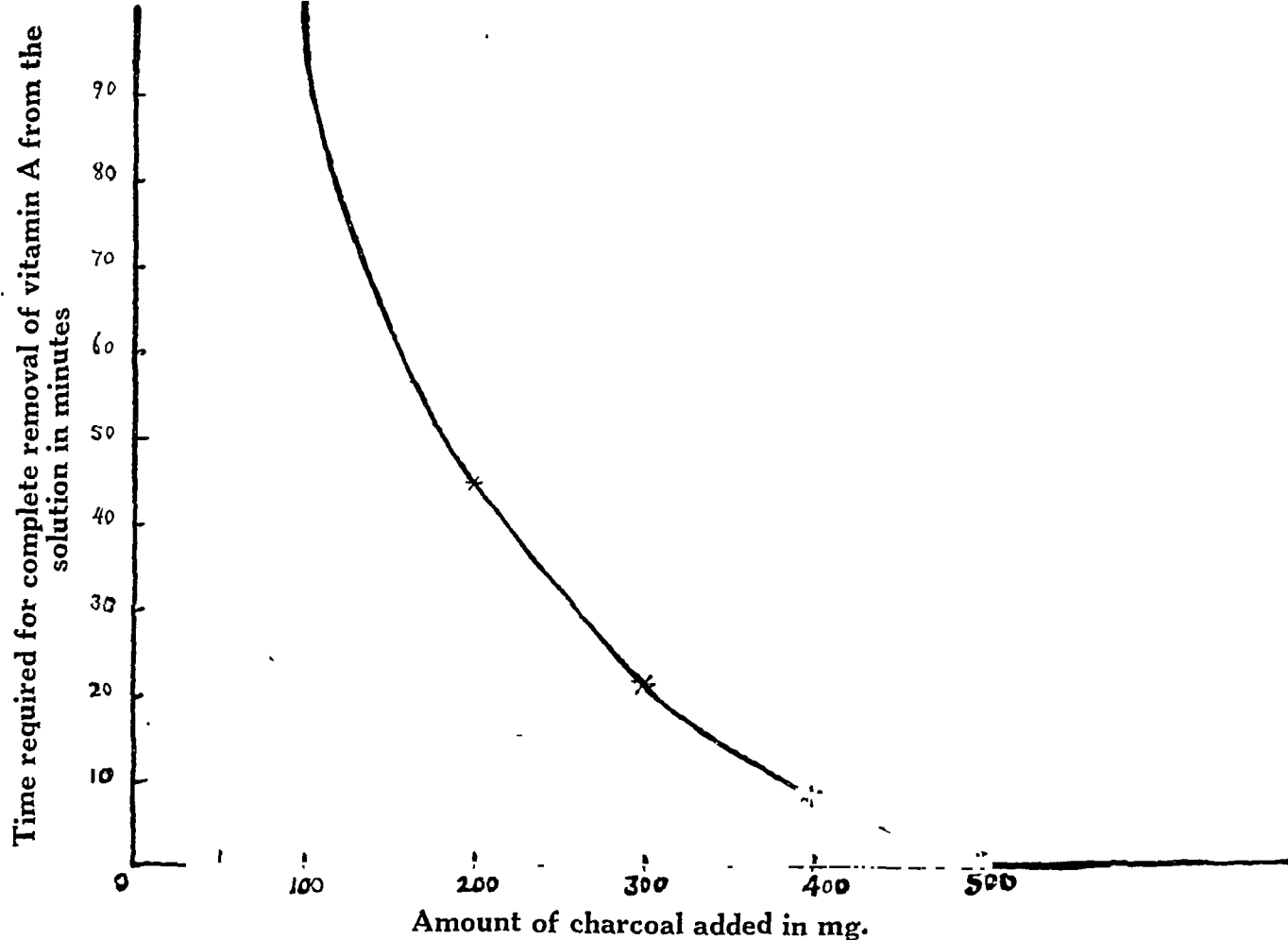


Fig. 4.—Showing how the time required for complete removal of vitamin A from solution varied according to the quantities of charcoal used. Ten-c.c. samples of the solution were treated with varying amounts of charcoal and shaken for different periods to determine the minimum time required for complete removal of vitamin A from solution (*cf.* Table VI).

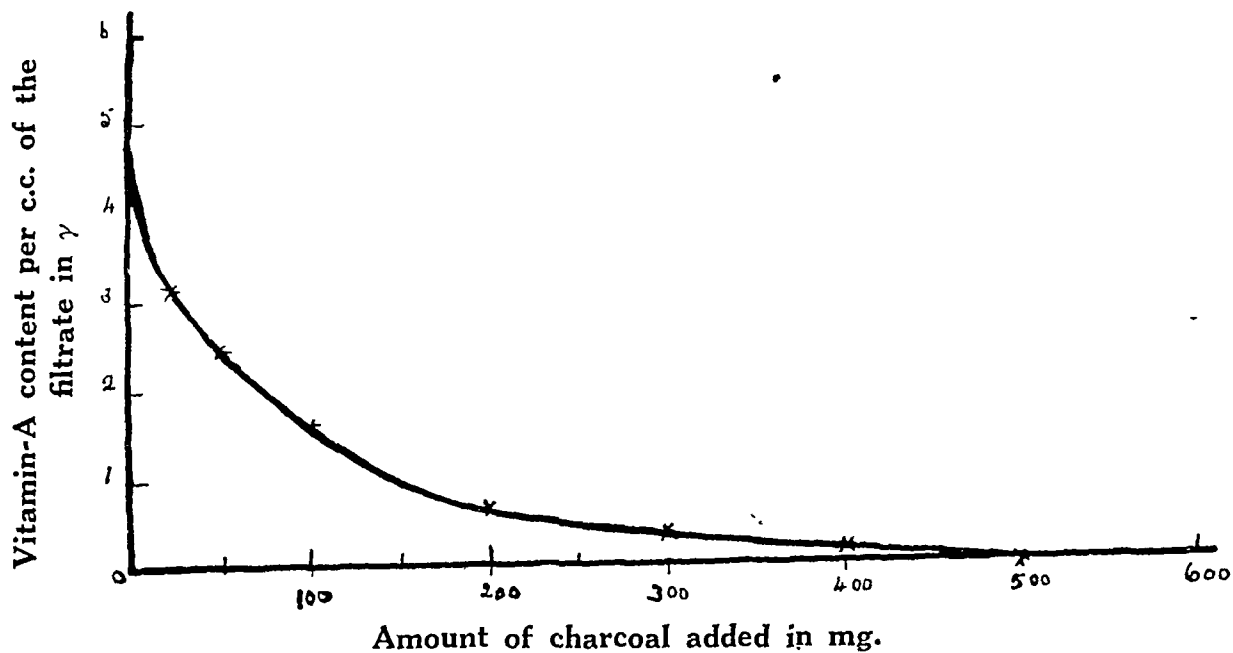


Fig. 5.—Showing how the adsorption of vitamin A varied according to the quantities of charcoal used. Ten-c.c. samples of the solution were treated with varying amounts of charcoal, each shaken for 3 minutes and filtered immediately (*cf.* Table VII).

The main problem was to find a means of removing carotene (or other pigments which are usually associated with vitamin A in natural foods) without any destruction of vitamin A. From Table VIII it will be seen that the charcoal-adsorption method was not satisfactory when applied to the unsaponifiable fractions, or even fat-poor solutions, since there occurred considerable loss of vitamin A (20 to 70 per cent). The loss must of course depend on the quantity of charcoal added and the time of shaking, etc.; but when the method was applied on crude solutions (containing large quantities of free fat) the loss of vitamin A was nil or very little, 0 to 6 per cent, even when the proportion of carotene was as much as 16 times that of vitamin A. It can be noted here that the amounts of vitamin A and carotenoid pigments likely to occur in most of the common animal products will rarely exceed this proportion. With smaller quantities of charcoal much longer periods of shaking were required for decolorization and as a result of such prolonged treatments part of the vitamin A was lost from the solution as evidenced by the SbCl_3 test.

DISCUSSION.

Vitamin A as such does not occur in vegetable products which contain only carotene. The two potent factors, vitamin A and carotene, occur simultaneously only in animal material (Wolff *et al.*, 1930; Moore, 1931; De, 1935). Hence in assaying the vitamin-A activity of such materials, particularly the rich sources, e.g., egg-yolk, butter, etc., it is desirable to estimate vitamin A and its precursor carotene separately. Chemical reactions given by vitamin A and carotene are more or less alike; vitamin A does not affect the specific absorption band of carotene in the visible region; while, on the other hand, carotene (like xanthophylls and many other similar pigments) may markedly influence the gross absorption at 3,280 A. U., which is taken as a physical criterion of vitamin A. Assay of carotene in general is simple or less complicated, while difficulties arise with vitamin A. To solve this difficulty two courses could be followed: (1) to deduct from the gross absorption at 3,280 A. U. that contributed by the carotenoid pigments; or (2) to devise a simple chemical process that will isolate vitamin A from the interfering pigments. Results of some attempts to solve this problem have been reported in a previous communication (De, 1937). It was observed that the first procedure and the process of phase separation (between alcohol and petrol) proved unsatisfactory; while preliminary observations on charcoal-adsorption methods gave encouraging results. A thorough examination of the last-named method has proved it to be efficient and satisfactory.

Results of the present investigation have shown that when the charcoal adsorption of carotene is carried out on crude solutions containing large quantities of free fat (with a minimum amount of charcoal and as quickly as possible), the loss of vitamin A from the solution will be nil or negligible. In assaying the vitamin-A content of an animal product, decolorization of the pigments should therefore be first carried out, under precautions as stated above, before saponification is resorted to. With test materials (like butter and egg-yolk), which can be obtained in the form of fluids or which go easily into solution in alcohol-ether or petrol, charcoal adsorption can be applied directly. In other cases (e.g., flesh foods), the materials should be finely ground and extracted with suitable solvents. The total extract

can then be decolorized by the charcoal and used for vitamin-A assay. It is important to note that if the test solutions are very poor in free fat, some vitamin-A-free oil should be added before charcoal adsorption is carried out.

To check the validity of the above suggestion, 60 γ to 100 γ of vitamin A and 1,000 γ of carotene were added to 3 c.c. to 10 c.c. of a vitamin-A-free coco-nut oil and the whole dissolved in 25 c.c. of ether (for easy filtration). The solution was decolorized by shaking for about 2 minutes with 1 g. to 2 g. of charcoal filtered immediately, and the precipitate washed 4 to 5 times with 5-c.c. fractions of fresh ether. The total filtrate was then dried up by low heat in absence of air, saponified, and its vitamin-A content determined spectrographically. The amount of vitamin A recovered in 5 experiments lay between 95 and 100 per cent. The maximum discrepancy of 5 per cent lies within the experimental error and could therefore be overlooked.

SUMMARY.

1. The relative adsorbability of vitamin A and carotene from different solvents and by different solid materials has been studied. Of all the agents used, only charcoal was a powerful adsorbent. It was noted that carotene is much more easily adsorbed than vitamin A, and that the degree of adsorption depends on the nature of the solvent, adsorption being more rapid from solvents like ether, petrol-ether, and hexane, etc., than from chloroform. It was also observed that the presence of free fat retards the adsorption considerably. The variation of the adsorption of vitamin A and carotene according to the quantities of free fat in solution, amounts of charcoal used, time and rate of shaking, etc., is described.

2. The investigation has revealed that carotenoid pigments can be completely removed from crude vitamin-A solutions by adsorption on charcoal without destroying vitamin A. This procedure, with due precautions, was found to be satisfactory for use in routine work.

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THE EXCRETION OF VITAMIN C BY HUMAN BEINGS IN SOUTH INDIA.

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HARRIS AND RAY (1935) have suggested that a considerable degree of 'vitamin-C sub-nutrition' may exist without any obvious clinical symptoms. These workers devised a chemical method of assessing such sub-nutrition, which consists of the determination of the daily urinary excretion of vitamin C, and the response to a large test dose of the pure vitamin, now easily available on the market. The rationale of the method is that if the body tissues are getting vitamin C in adequate amounts, or are, in other words, 'saturated' with the vitamin, large test doses administered orally are largely excreted in the urine within 24 hours of ingestion; if, however, the body tissues are not so 'saturated', the extra vitamin supplied is utilized quickly by the tissues, and its excretion does not rise. Abbasy, Harris, Ray and Marrack (1935), Archer and Graham (1936*a* and *b*), Hawley *et al.* (1936), and Harris *et al.* (1936) have employed this method to assess the extent of vitamin-C sub-nutrition in apparently 'normal' subjects. In South India intake of vitamin C in the form of fresh fruit and vegetables is in general low, and it was felt that an investigation of vitamin-C excretion under experimental conditions would throw light on the problem of vitamin-C sub-nutrition.

EXPERIMENTAL.

Harris and Ray's technique for the determination of the urinary excretion of ascorbic acid during the 24 hours before and after administration of a test dose of the vitamin was employed with slight modifications. Abundant evidence exists to show that this vitamin is not excreted to any great extent in the faeces, even when large test doses are administered; also in a 'saturated' individual there is no appreciable storage of the vitamin in the system. The level of excretion in

the urine is, therefore, a good index of the individual's response to test doses of the vitamin.

Estimations of ascorbic acid in the urine were carried out by the standard chemical method, employing Tillman's indicator, 2, 6-dichlorophenol indophenol. They were mostly done on freshly voided specimens of urine, but where this was impossible, as in the case of specimens voided during the night, the urine was passed into bottles containing enough glacial acetic acid to make a final concentration of about 10 per cent acid, and titrated early in the morning. The glacial acetic acid, which proved to be a better preservative of ascorbic acid than other substances suggested for this purpose, minimized the destruction of the vitamin in the voided urine. Further, the experiments were carried out in Coonoor during the coldest season of the year, when little deterioration of urine in a few hours would normally take place.

The present investigation covered 34 subjects, the majority of whom were Indians living in Coonoor. Boys and girls between 5 and 13 years of age, adult males between 16 and 35, a few elderly men and women over 60, pregnant women and lactating mothers were included. In addition a number of British and Sikh soldiers were studied. The latter were healthy subjects housed in hospitals having an adequate nursing staff and were kept resting during the investigation. The hospitals were visited at four-hourly intervals during the day and ascorbic acid titrations carried out on freshly voided urine in a medium of strong acetic acid. The volume of urine passed was recorded.

Not more than four or five subjects were investigated at the same time. When the initial level of urinary excretion of ascorbic acid during 24 hours had been determined, a large test dose of ascorbic acid, usually in the form of the pure substance but sometimes as both ascorbic acid and fresh orange juice, was administered orally. The weighed amount of the vitamin-C powder was swallowed with a little drinking water; though it is sour, the subjects were not averse to taking it. When orange juice was given its ascorbic-acid content was determined immediately. During the experiments the subjects consumed their normal diet; any unusually large addition of fruits or green vegetables, likely to increase the intake of the antiscorbutic vitamin, was guarded against. In the majority of cases strict supervision of the food intake was possible. After the administration of the test dose, determinations of ascorbic acid in urine were done as before during the following 24 hours. The results showing the initial level of urinary excretion during 24 hours, the response to test doses, volume of urine voided, etc., are set out in Table I.

It is seen from Table I that only two (cases 7 and 32) out of the 34 subjects in the different age groups and communities examined responded to the first test dose by immediate excretion. Case 32, a nursing orderly in a neighbouring Military Hospital, showed an initial urinary excretion of 20.36 mg. of ascorbic acid per diem, a figure exceeded by two other members of his group; yet, on administration of a 300 mg. test dose, only this case showed a response and excreted 133.09 mg. or 44.36 per cent of the amount ingested during 24 hours. Case 7 showed a response with a test dose of 150 mg. of ascorbic acid, excreting 18.94 per cent of the intake in the 24 hours following the administration of the test dose.

The average daily excretion in the 34 cases examined was 8.65 mg.; the Sikh and the British troops, exhibiting a higher level of urinary excretion, had an average of 18.01 mg., while the remainder of the population showed an average of only 4.75 mg. of ascorbic acid.

Harris *et al.* (*loc. cit.*) have reported that 84 per cent of hospital patients in Cambridge excreted less than 13 mg. of ascorbic acid, which they regarded as the minimum excretion indicating a satisfactory intake of the vitamin. It thus appears that 'hypovitaminosis C', in so far as it is demonstrated by uninfluenced levels of excretion, is almost as common in English people of the poorer classes as in the groups studied in our investigation.

TABLE I.

Excretion of vitamin C before and after administration of test doses.

Case number.	Name.	Initial level of vitamin-C excretion in urine in 24 hours in mg.	Volume of urine voided in this 24 hours in c.c.	Amount of the test dose of vitamin C in mg.	RESPONSE TO TEST DOSES.		REMARKS.
					Excretion of vitamin C in urine in 24 hours in mg.	Volume of urine voided during this 24 hours in c.c.	
1	K. R. ..	9.38	2,945	150.0	8.76	2,500	'Normal' adults between 25 and 35 years; the test dose was partly pure ascorbic acid and partly fresh orange juice.
				327.0	6.77	1,890	
2	M. S. ..	7.73	2,975	150.0	6.82	2,500	
				327.0	4.62	1,180	
3	S. R. ..	7.75	2,260	150.0	11.72	2,665	
				327.0	5.63	1,390	
4	G. S. ..	7.59	1,580	150.0	7.45	1,750	'Normal' boys and girls between 5 and 13 years; the test dose was pure ascorbic acid only.
				327.0	8.67	1,470	
5	S. ..	1.97	1,105	150.0	1.29	1,090	
6	V. ..	1.20	610	150.0	1.47	410	
7	Mi. ..	7.92	1,205	150.0	23.41	690	
8	E. ..	2.53	1,020	150.0	1.79	875	
9	P. ..	2.31	1,560	150.0	3.15	810	

TABLE I—*contd.*

Case number.	Name.	Initial level of vitamin-C excretion in urine in 24 hours in mg.	Volume of urine voided in this 24 hours in c.c.	Amount of the test dose of vitamin C in mg.	RESPONSE TO TEST DOSES.		REMARKS.
					Excretion of vitamin C in urine in 24 hours in mg.	Volume of urine voided during this 24 hours in c.c.	
10	B. ..	7.72	2,190	101.6*	2.29	755	<p>'Normal' pregnant women; the test dose was pure ascorbic acid, except in the two cases marked *. In these, the dose was partly pure ascorbic acid and partly fresh orange juice.</p>
				150.0	2.58	1,835	
11	Me. ..	4.78	1,595	101.6*	0.88	305	
				150.0	1.79	1,115	
12	S. ..	1.68	1,475	150.0	1.82	1,340	
				300.0	1.17	685	
13	K. ..	1.35	515	150.0	2.85	1,820	
				300.0	0.48	270	
14	R. ..	2.88	1,245	150.0	3.05	2,060	
				300.0	3.47	845	
15	Lak. ..	4.47	1,450	300.0	7.23	1,420	<p>'Normal' lactating mothers; the test dose was pure ascorbic acid only.</p>
16	Mary ..	6.35	1,660	300.0	6.07	1,650	
17	Marim.	5.72	545	300.0	5.84	840	
18	Cl. ..	6.63	1,370	300.0	6.42	1,555	
19	Kull. ..	4.60	895	300.0	4.52	1,220	
20	Kan. ..	6.36	955	300.0	8.15	1,630	<p>Elderly men and women of over 60 years; the test dose was pure ascorbic acid only.</p>
21	Sham. ..	3.89	1,460	300.0	4.49	1,385	
22	Ran ..	5.31	1,390	300.0	6.74	1,485	
23	Mich. ..	1.68	190	300.0	0.83	415	
24	Mee. ..	2.29	820	300.0	1.73	995	

TABLE I—concl'd.

Case number.	Name.	Initial level of vitamin-C excretion in urine in 24 hours in mg.	Volume of urine voided in this 24 hours in c.c.	Amount of the test dose of vitamin C in mg.	RESPONSE TO TEST DOSES.		REMARKS.
					Excretion of vitamin C in urine in 24 hours in mg.	Volume of urine voided during this 24 hours in c.c.	
25	H. S. ..	15.86	1,525	300.0	15.85	850	Sikh soldiers attached to the Wellington Military Station; the test dose was pure ascorbic acid only.
26	B. S. ..	16.87	1,040	300.0	18.05	1,270	
27	S. S. ..	13.41	1,695	300.0	18.72	1,660	
28	J. S. ..	21.88	1,330	300.0	14.86	1,040	
29	K. S. ..	20.36	1,150	300.0	18.06	1,345	
30	Wi. ..	11.22	1,425	300.0	11.15	1,375	British soldiers in the Military Hospital, Wellington; the test dose was pure ascorbic acid only.
31	C. ..	10.81	1,205	300.0	13.66	1,025	
32	Mac. ..	20.36	950	300.0	133.09	1,405	
33	W. ..	22.63	1,785	300.0	21.68	1,705	
34	M. ..	27.00	1,570	300.0	17.16	965	

It may be seen from the above table that the initial level of vitamin-C excretion in the urine does not necessarily provide an index of the degree of 'hypovitaminosis C'; to assess the latter by this method, it is necessary that the response to test doses should be determined. Thus, while case 7 showed 'saturation' with vitamin C even on an initial urinary excretion of 7.92 mg., cases 1, 25 to 31, 33, and 34, with much higher initial levels of excretion, showed no response to the test dose.

VITAMIN-C 'SATURATION' EXPERIMENTS.

These investigations suggested that vitamin-C sub-nutrition is common in South India. A further series of experiments was undertaken to study the 'saturation' point in two subjects. To these, progressively increasing doses of ascorbic acid were administered. The excretion of ascorbic acid in the urine, before and after the ingestion of test doses, was determined on fresh specimens by the method previously described. During the course of these 'saturation' experiments, the subjects consumed their usual diet.

TABLE II.

Vitamin-C 'saturation' experiments.

	Excretion of vitamin C in 24 hours in mg.	Volume of urine voided in 24 hours in c.c.	Percentage of test dose ex- creted in urine in 24 hours.
<i>Case 3, S. R.</i>			
Initial level ..	7.75	2,260	..
After 150 mg. of vitamin C. (Pure ascorbic acid and orange juice.)	11.72	2,665	7.80
After 327 mg. of vitamin C. (Pure ascorbic acid and orange juice.)	5.63	1,390	1.70
After 1,136 mg. of vitamin C. (Pure ascorbic acid and orange juice.)	170.42	2,785	14.70
After 335 mg. of vitamin C. (Pure ascorbic acid and orange juice.)	178.01	2,850	53.10
After 130 mg. of vitamin C. (Pure ascorbic acid and orange juice.)	118.29	2,125	91.00
After 50 mg. of vitamin C. (Pure ascorbic acid only.)	44.70	2,185	88.90
After 25 mg. of vitamin C. (Pure ascorbic acid only.)	30.44	1,900	> 100.00
<i>Case 25, H. S.</i>			
Initial level ..	15.86	1,525	..
After 300 mg. of vitamin C. (Pure ascorbic acid only.)	15.85	850	2.80
After 1,250 mg. of vitamin C. (Pure ascorbic acid only.)	128.44	1,260	10.28
After 100 mg. of vitamin C. (Pure ascorbic acid only.)	65.89	1,325	65.89

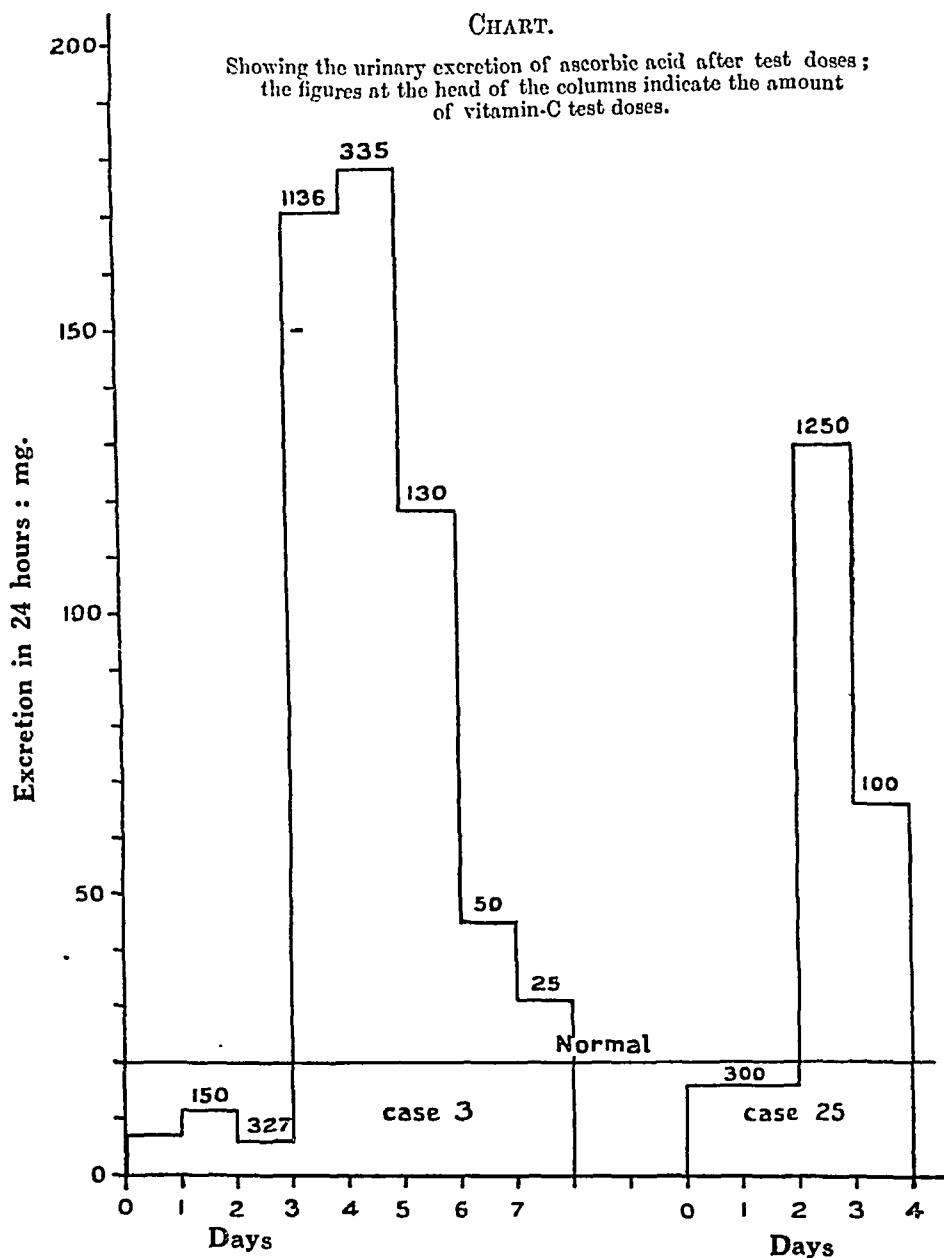
It will be seen that, irrespective of the initial level of the excretion in the urine, not until doses exceeding 1,000 mg. of the vitamin were administered was there observed a 'peak' in the urinary excretion. In case 3, the body tissues became 'saturated' after the dose of 1,136 mg. Though the 'peak' was observed at this point, the excretion of ascorbic acid in the 24 hours following this dose was only 14.7 per cent of the intake. Once the tissues were 'saturated', smaller test doses were to a great extent excreted in the urine. Thus after 'saturation' 53.1 per cent of a test dose of 335 mg. of vitamin C were excreted in the urine within 24 hours. Subsequent daily doses of 130 mg., 50 mg., and 25 mg. were excreted at 91.0, 88.9, and over 100 per cent levels, respectively. The excess excretion of vitamin C over intake during the last day may presumably be accounted for by the vitamin C present in the usual diet or perhaps by residual ascorbic acid left unexcreted during the previous 24 hours when higher doses were being administered.

In the second case (case 25), 65.89 per cent of the intake were excreted after 'saturation' with 1,250 mg. Before saturation, after a test dose as high as 300 mg., the urinary excretion was not very different from the initial level determined before administration of any test dose of the vitamin. The results are graphically represented in the Chart.

A rough quantitative measure of 'vitamin-C sub-nutrition' might be derived from 'saturation' experiments. Presumably, if an individual is already

CHART.

Showing the urinary excretion of ascorbic acid after test doses ;
the figures at the head of the columns indicate the amount
of vitamin-C test doses.



'saturated', any additional ascorbic acid given as a test dose should be excreted apart from whatever is destroyed in the system. In case 3 the 'peak' of

excretion occurred in three days, after 1,613 mg. of vitamin C had been taken. During this period, 195.5 mg. were excreted, representing 12.12 per cent of intake. The degree of 'vitamin-C sub-nutrition' in this case might be represented as 87.88 (100-12.12). Similarly, in case 5, with an intake of 1,550 mg. at the first peak and an excretion of 10.33, the degree would be described as 89.67. The hypothetical figure 100 would represent a maximum degree of 'sub-nutrition', and the equally hypothetical figure 0 complete 'saturation'. Destruction of vitamin C in the body must, of course, vary with individuals, and this might introduce fallacies in the above method of assessment. Nevertheless, it might be useful for comparative purposes.

EFFECT OF INTAKE OF VITAMIN C ON ITS SECRETION IN HUMAN MILK.

In the present investigation six lactating mothers who were apparently in normal health were included. They were kept under observation, three at a time; the second group of three mothers was perhaps in a better condition than the first group, being drawn from a slightly higher social stratum. In these cases, in addition to urinary determinations, the vitamin-C content of the breast milk, before and after the administration of the test doses of the vitamin, was investigated. Three determinations were carried out at different intervals of time after the test dose.

The ascorbic-acid content of milk was determined as follows: enough trichloroacetic acid was added to a known volume of freshly drawn milk to produce a concentration of 8 to 10 per cent acid in the final medium. The mixture was filtered through dry filter-paper into a dry flask, aliquots being titrated against standard indophenol solution as quickly as possible. Blanks were investigated to test the quality of the reagents employed, particularly trichloroacetic acid. The results of the determinations on the six lactating mothers are shown in Table III:—

TABLE III.

The vitamin-C content of human milk before and after administration of test doses of vitamin C.

Name.	Initial content in mg.	VITAMIN-C CONTENT IN MG. AFTER ADMINISTRATION OF 300 MG. OF ASCORBIC ACID.		
		After 8 hours.	After 24 hours.	After 30 hours.
Lakshmi ..	0.10	1.75	1.89	0.98
Mary ..	0.10	2.98	3.13	1.34
Marimuthu ..	0.15	2.52	2.33	1.12
		After 4 hours.	After 8 hours.	After 24 hours.
Clara ..	1.15	1.30	1.10	1.32
Kullamma ..	0.95	1.63	1.68	1.40
Kānnamma ..	2.41	2.45	1.86	1.96

The results show that the first group of three mothers, with a low content of vitamin C in their milk, displayed a quick response to the test dose of the vitamin, while in the second group in which the milk was richer in vitamin C the effect was not perceptible. The response to test doses was more rapidly apparent in the milk than in the urine. The effect of the test doses of the vitamin extended to periods beyond 30 hours.

DISCUSSION.

In this paper the term 'vitamin-C sub-nutrition' has been used in connection with a particular test. It must be emphasized that we have at present no clinical or other data to show that subjects with a low excretion of vitamin C are necessarily in a poor state of health and nutrition. The physical effects of a low vitamin-C intake must be the result of further investigation. Gingivitis, periodontal infections, and pyorrhœa alveolaris are very common in South India, and there may be some relation between these conditions and a relatively small intake of fresh fruits and vegetables. The importance of an adequate intake of vitamin C has been stressed by many workers. Mention may be made of the following investigations: vitamin C in relation to certain teeth affections (Fish and Harris, 1934); to the healing of gastric and duodenal ulcers (Archer and Graham, 1936a); to the maintenance of the glucose-tolerance level (Sigal and King, 1936); protection against anaphylactic shock (Solomonica, 1936); improvement of the blood picture without iron and liver modification (Archer and Graham, 1936b). All these investigations go to show that the vitamin-C intake of a population is an important question requiring careful investigation. The present study certainly suggests that hypovitaminosis C may be a common deficiency in South India.

ACKNOWLEDGMENTS.

Grateful acknowledgments are due to the authorities of the Lawley Hospital, Coonoor, British Military Hospital, Wellington, and Child Welfare Centre, Coonoor, for affording us all facilities to carry out these experiments.

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STUDIES ON VITAMIN-A DEFICIENCY.

Part II.

HISTOPATHOLOGY OF THE SKIN IN HUMAN KERATOMALACIA.

BY

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It is now generally accepted that keratomalacia is a disease-complex manifesting symptoms not only in the eye but also in several parts of the body. Most workers on keratomalacia who did not limit their clinical observations to the eye have described changes in the skin as part of the syndrome. Thus, de Gouvêa (1883), Bloch (1917, 1921), Wilson and du Bois (1923), Blackfan and Wolbach (1933), and others reported the occurrence in infants of a dry, harsh, and scaly condition of the skin in association with the ocular manifestations. A peculiar dry, earthy coloration of the skin has been described by Wright (1922, 1933-34, 1936), Elliot (1920), Herbert (1897), Hsu (1927), Stephenson (1912), and others. Pillat (1929*a* and *b*), who gave a detailed account of cutaneous changes in adult keratomalacia, pointed out that in severe cases the skin of the whole body felt dry 'as if it had been strewn with coarse powder'. The literature on the subject was briefly reviewed by Mackay (1934) and Frazier and Hu (1936). In spite, however, of the early recognition of the existence of changes in the skin and the general agreement that they form part of the syndrome of keratomalacia, no detailed investigation of the histopathology of the skin condition has hitherto been made.

During recent years a papulo-follicular dermatosis called 'phrynoderma' (Nicholls, 1933) has been described in association with dry skin in cases of keratomalacia (Nicholls, *loc. cit.*; Frazier and Hu, 1931, 1936; Loewenthal, 1933*a* and *b*; Wright, 1936). In a previous communication (Radhakrishna Rao, 1937) the clinical and histopathological features of this papular eruption were described, and it was pointed out that further investigation was necessary to establish the exact ætiology of the condition, though the morphological appearances resemble those seen in vitamin-A deficiency. In the present investigation changes in the

skin in advanced cases of keratomalacia, not showing the follicular eruption or secondary infections, have been studied.

MATERIAL.

Clinical observations of the condition of the skin in cases of keratomalacia in the Government Lawley Hospital, Coonoor, confirm the observations of previous workers and need no lengthy description here. In one advanced case in a woman, the skin of the whole body was dry, inelastic and dull grey in colour; with the exception of the face, the body was covered with scales, and scratching produced a white powdery desquamation.

Skin clips for histopathological study were obtained by biopsy from the Government Ophthalmic Hospital, Madras, and the Government Lawley Hospital, Coonoor, the majority from the former. These were taken from the extensor aspect of the arms of 15 typical cases showing advanced keratomalacia. All except three were adult cases. Most of the patients in this series gave a history of having had night blindness, diarrhoea or dysentery, fever, and cough; some of them showed an enlargement of the liver, or spleen, or of both.

Histopathology.

Technique.—Pieces of skin were embedded in paraffin, and serial sections were stained by Ehrlich's acid-hæmatoxylin and eosin, orcein, and Weigert's iron-hæmatoxylin and van Gieson's stain.

(a) *Epidermis.*—Most of the cases showed a superficial hyperkeratosis of the epidermis (Plate III, figs. 6 and 7). There was an excessive formation of horn-cells in these cases without any signs of parakeratosis; occasionally, however, small masses of horn-cells with persistent nuclei were seen on the surface of the epidermis. The *stratum corneum*, which was broadened, appeared as a loose network (Plate III, figs. 6 and 7). In one case, however, in which the skin was atrophic and presented a cracked appearance (Plates I and II, figs. 1 to 3), the horny layer was greatly enlarged, homogeneous in structure and transformed into a horny mass (Plate IV, figs. 8 and 9); there was an imperfect cornification of the horn-cells (parakeratosis) and in places collections of red blood cells and leucocytes were seen in between the layers of keratin (Plate IV, figs. 10 and 11).

In some instances the epidermis was thinner than normal (Plate III, fig. 6). Granules of melanin pigment were increased in the cells of the *basal layer* of the epidermis, and, in some places, the pigment-bearing cells were increased in number and were present throughout the thickness of the *rete mucosum*. Acanthosis of the prickle-cell layer is not a marked feature, though in some instances short processes were seen dipping down into the corium.

The cells of the *stratum lucidum* and *stratum granulosum* showed no appreciable changes.

(b) *Corium (cutis vera).*—In cases in which the epidermis showed atrophic changes, the papillary layer was flattened out. Perifollicular infiltration—mostly fibroblasts and mononuclear lymphoid cells—was limited to those hair follicles in which marked changes were evident.



FIG. 1.



FIG. 2.

Figs. 1 and 2.—Photographs showing the dry and atrophic skin in a man aged 20 years with keratomalacia. Note the cracked appearance of the skin and the scales covering the body. The condition was labelled 'Giraffe skin' by Lieut-Colonel R. E. Wright, who kindly supplied the

skin in advanced cases of keratomalacia, not showing the follicular eruption or secondary infections, have been studied.

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PLATE III.

[All the photomicrographs were taken with 'Miflex' (Zeiss).]



FIG. 4.

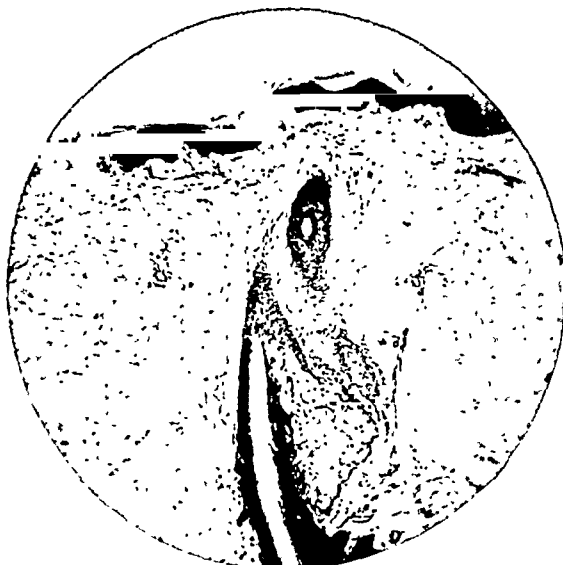


FIG. 5.

Figs. 4 and 5.—Sections of skin from a labourer, aged 25 years, apparently in normal health. The skin clipping was taken to serve as a control, and the sections illustrate normal appearances. $\times 50$.

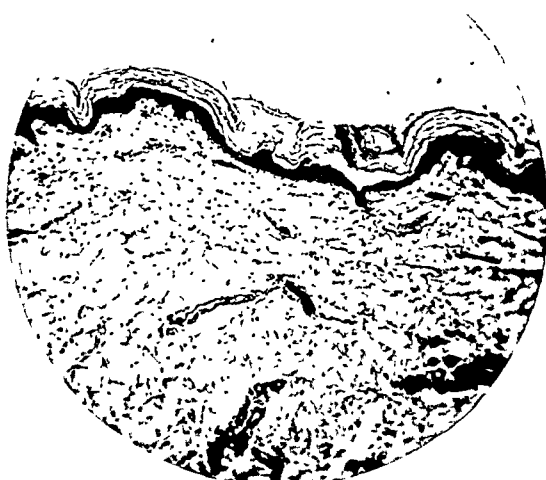


FIG. 6.

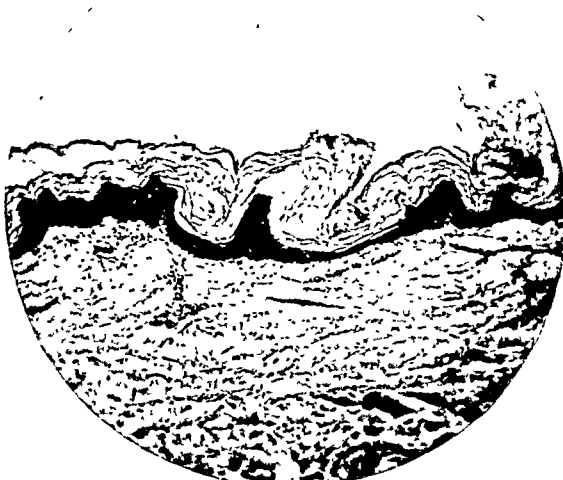


FIG. 7.

Figs. 6 and 7.—Superficial hyperkeratosis of the epidermis in keratomalacia. The horny layer is broadened and appears as a loose network. The epidermis is thinner than normal in Fig. 6. $\times 50$.

PLATE IV.



FIG. 8.

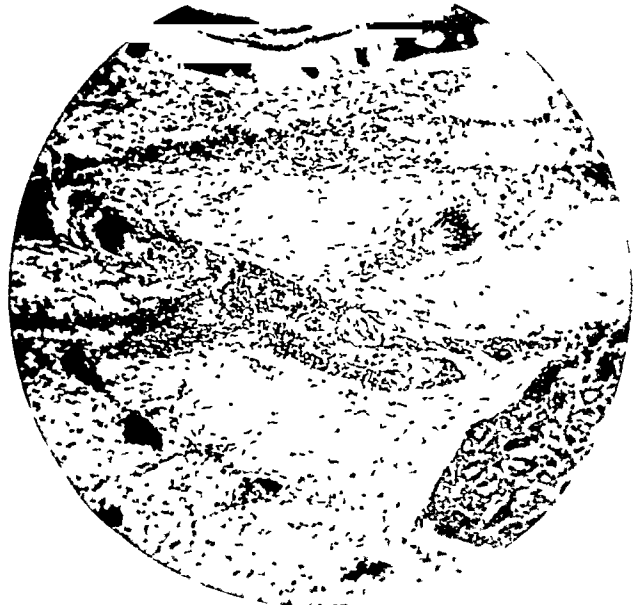


FIG. 9.

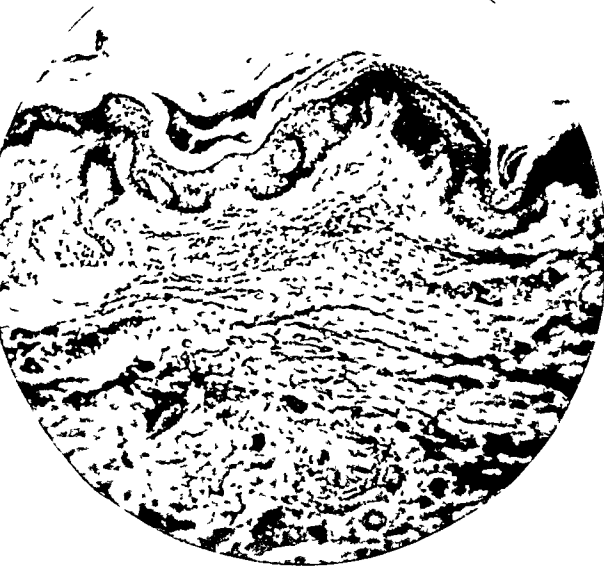


FIG. 10.

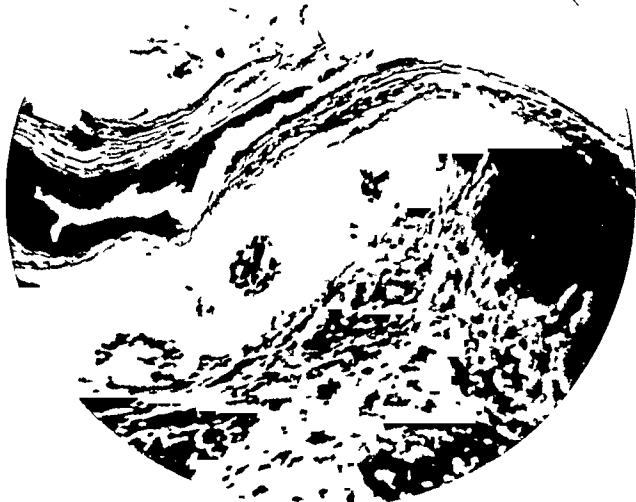


FIG. 11.

Figs. 8 to 11.—Sections of skin from the patient shown in Figs. 1 to 3. Note the homogeneous structure of the *stratum corneum*, the parakeratosis (Figs. 10 and 11), and the perivascular lymphocytic infiltration and the large multinucleated cells (Fig. 9) in the corium. Fig. 8 shows a dilated hair follicle and the coiled and atrophic hair in the substance of the follicular plug. Note the collection of cells in between the layers of *stratum corneum* in Figs. 10 and 11.

(Figs. 10 and 11.—Weigert's iron-haematoxylin and van Gieson's stain). Figs. 8 to 10 $\times 50$, Fig. 11 $\times 100$.

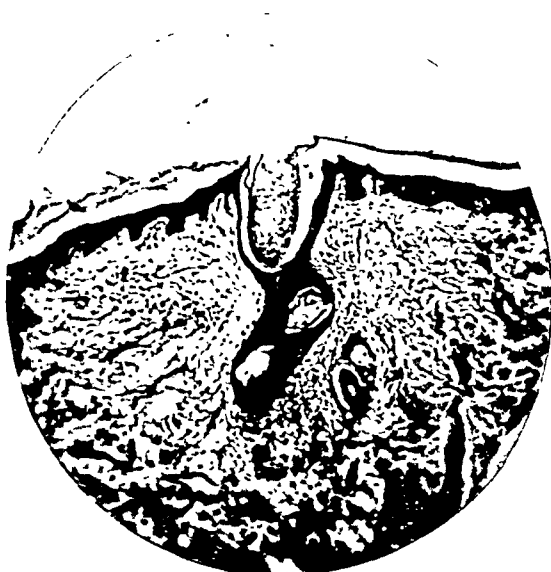


FIG. 12.

Fig. 12.—Section of scalp showing an atrophic hair follicle. Note the cellular infiltration in the perifollicular tissues. $\times 50$.

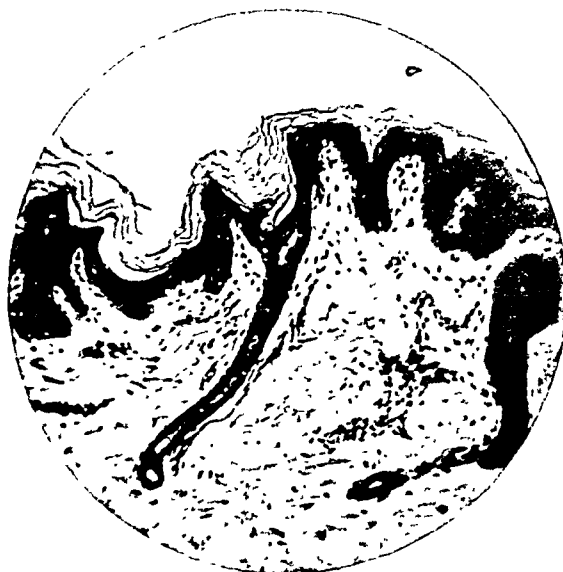


FIG. 15.

Fig. 15.—A sweat duct showing occlusion of its opening with keratinous material. $\times 100$.



FIG. 13.



FIG. 14.

Figs. 13 and 14.—Hair follicles showing varying degrees of hyperkeratinization of the lining epithelium. Fig. 13 $\times 10$; Fig. 14 $\times 50$.

In the case illustrated in Plates I and II, figs. 1 to 3, there was marked perivascular lymphocytic infiltration. In places, large multinucleated cells, with nuclei arranged in a small group in the centre of the cell, were seen in the cellular infiltration (Plate IV, fig. 9). A similar accumulation of cells was also seen around the sweat glands. The collagen bundles and the elastic fibres in the corium were separated as a result of the cellular infiltration; apart from this, the fibrous and elastic elements appeared normal.

Few pigment cells were seen in the papillary layer of the corium.

(c) *Sebaceous glands*.—Marked changes were found in the sebaceous glands in all instances. The glands were completely absent in two cases including the one referred to above (Plates I and II, figs. 1 to 3); in others, they were few in number. The sebaceous glands associated with the hair follicles which showed the follicular lesions appeared small and atrophic; the glandular cells were diminished in number and their cytoplasm was decreased in amount.

(d) *Sweat glands*.—Apart from the cellular infiltration around the sweat glands in one case, mentioned above, no appreciable changes were found in the sweat glands. Slight dilatation of the lumen of the coil glands, with a flattening of the lining epithelium, was, however, occasionally seen. The funnel-like openings of some of the sweat ducts were occluded to a varying extent with keratinous material (Plate V, fig. 15).

(e) *Hair follicles and hair*.—The superficial hyperkeratosis of the epidermis extended, in many instances, into the mouths of the pilo-sebaceous follicles (Plate IV, fig. 8; Plate V, figs. 13 and 14). The epithelium lining the follicles showed hyperkeratinization, and, in some instances, there was a tendency for the formation of horny plugs resulting in a widening of the follicular ostium. Similar changes were seen in the scalp in one case in which a piece was removed for examination (Plate V, fig. 12). Parakeratosis was evident in the outer layers of some of the follicular plugs. The remains of the hair were generally seen, often coiled up, in the horny mass of the dilated follicles. Atrophic changes were rarely found in the lower part of some of the hair follicles showing the above-mentioned follicular lesions.

DISCUSSION.

The principal microscopic changes in the skin in keratomalacia consist of a superficial hyperkeratosis of the epidermis extending into the mouths of the pilo-sebaceous follicles, slight but demonstrable atrophy of some of the hair follicles and atrophic changes in the sebaceous glands. In some instances, the epidermis was thinner than normal. The exact sequence of these changes in the skin is difficult to judge from a study of the late stages of the skin condition as seen in the present series of cases; a study of serial skin sections in these cases, however, suggests that the hyperkeratinization of the epidermis is the primary feature, while the atrophic changes in the sebaceous glands are either secondary or coincident. No exact parallelism exists between the degree of hyperkeratosis of the epidermis and the intensity of the changes present in the sebaceous glands.

The histopathological features as described indicate that the rough and dry skin seen clinically in cases of keratomalacia is due to an excessive epithelial

keratinization and an absence or diminution of the secretions of the cutaneous glands. The absence of marked sweating in these cases is probably due to a loss of the specific function of the epithelium of the coil glands which, as in the present series of cases, may not show any demonstrable pathological lesions. Wolbach and Howe (1925) concluded that in experimental avitaminosis-A in albino rats the deficiency results in a loss of the specific (chemical) functions of the affected secretory epithelium and in the substitution of a chemically inactive non-secretory epithelium. These authors reported in vitamin-A-deficient rats skin changes similar to those described here. Atrophy of the sebaceous glands has been attributed by Bommer (1934) to a deficiency of vitamin A. The peculiar greyish or slaty coloration of the skin seen clinically in cases of keratomalacia is probably due to the increased pigment in the cells of the basal layer, and to an increase of the pigment-bearing cells in the epidermis. The wrinkling of the surface epithelium, consequent on its atrophy, may apparently increase the pigment per unit of body surface, and thus may contribute to the appearances seen clinically. Pigmentation of the conjunctiva in cases of keratomalacia has been described by Wright (1922), Mori (1924), Pillat (1929*a*, 1932), and others. The explanation of the increased pigmentation of these epithelial surfaces is far from clear. Pillat (*loc. cit.*) suggested that there is probably disease of the suprarenal gland. Further work on this problem is required.

Except in one case, inflammatory changes in the corium were not present. The cellular infiltration and the multinucleated cells in the corium in this case were probably secondary, due to a superimposed chronic infection.

The data obtained in this study provide evidence that the nutrition of the skin suffers in keratomalacia. The condition of the skin appears to be a dystrophy, the pathological changes being due to an interference with its nutrition. It is generally recognized that pure states of avitaminosis, as seen in experimental animals, are not encountered in human beings; human keratomalacia may, however, be assumed to resemble the pure avitaminosis-A of experimental animals (Wright, 1936). The patients in the present series were in a state of vitamin-A deficiency shown by the presence of keratomalacia. The presence of other minor nutritional deficiencies cannot be excluded in these cases, but obvious clinical signs of such deficiencies were absent. The histopathological changes described in this paper may therefore justifiably be ascribed to vitamin-A deficiency, and they conform to the morphological appearances described by Wolbach and Howe (*loc. cit.*), Goldblatt and Benischek (1927), and others in experimental avitaminosis-A. Whether similar histopathological lesions in the skin may occur as the result of other deficiencies remains to be investigated.

A striking similarity is evident between the microscopic lesions in the skin in keratomalacia, described above, and those described in 'phrynoderma' by Frazier and Hu (1931, 1934, 1936), Loewenthal (1933*a*) and Radhakrishna Rao (*loc. cit.*). The follicular lesions were, however, more marked in phrynoderma; hyperkeratinization of the lining epithelium of the follicles was more pronounced, and there was marked dilatation of the funnels of the pilo-sebaceous follicles by keratotic plugs. The histopathological differences in the hair follicles in the two conditions appear to be only one of degree and not of quality. Frazier and Hu (*loc. cit.*) and Loewenthal (1935) described a dry and rough skin preceding the onset of the papular lesions. Such a sequence of events may suggest that the

popular lesions represent a later stage of the dry and rough skin. If it is so, it would be interesting to study whether the papules are the result of a pathological response to external irritation (e.g., dirt), or whether they represent a localization of the pathological process resulting from defective nutrition to the funnels of the pilo-sebaceous follicles. Clinical investigation of the effect of vitamin-A concentrates on the papular eruption in 'phrynoderma' would help to elucidate the ætiology of the condition and the association between phrynoderma and the changes described here. Such an experiment is now in progress. The present investigation appears to support the view that 'phrynoderma' is associated with vitamin-A deficiency.

SUMMARY AND CONCLUSION.

The histopathological features of the skin in human keratomalacia were studied. The principal microscopic changes consist of a superficial hyperkeratosis of the epidermis extending into the mouths of the pilo-sebaceous follicles, atrophy of the sebaceous glands, and slight atrophic changes in some of the hair follicles.

It is shown that the above-mentioned lesions in the skin resemble those described in 'phrynoderma', and the advanced follicular lesions in the latter differ from the former only in degree and not in quality.

ACKNOWLEDGMENTS.

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TABLE I.

The composition of foodstuffs before and after cooking.

Foodstuffs.	MOISTURE.		PROTEIN.			ETHER EXTRACTIVES.			MINERAL MATTER.			CARBOHYDRATES.		
	Uncooked, per cent.	Cooked, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.
<i>Cereals—</i>														
Rice, raw, milled ..	15.31	73.89	7.36	6.71	-0.65	0.60	0.19	-0.41	0.80	0.27	-0.53	75.93	65.45	-10.48
Rice, parboiled, milled ..	15.49	75.88	7.88	7.65	-0.23	0.71	0.12	-0.59	1.48	0.29	-1.19	74.44	65.43	-9.01
Rice, raw, home-pounded.	12.69	77.59	7.86	7.68	-0.18	1.29	0.67	-0.62	1.22	0.56	-0.66	76.94	74.90	-2.04
Rice, parboiled, home-pounded.	12.50	80.52	7.66	7.40	-0.26	0.53	0.44	-0.09	1.34	0.56	-0.78	77.97	71.10	-6.87
Ragi ..	15.16	65.19	7.43	7.50	+0.07	1.43	0.73	-0.70	3.33	3.36	+0.03	72.65	73.47	+0.82
Cambu ..	12.61	71.88	10.04	10.28	+0.24	4.06	2.15	-1.91	2.97	3.00	+0.03	70.32	71.40	+1.08
Wheat ..	14.62	76.91	12.26	10.24	-2.02	2.23	0.69	-1.54	1.65	1.09	-0.56	69.60	64.50	-5.10
Cholam (jowar) ..	14.45	69.66	9.79	9.87	+0.08	3.79	2.09	-1.70	1.68	1.68	0	70.29	72.10	+1.81
<i>Pulses—</i>														
Red gram (dhal arhar)	13.08	65.16	21.90	19.58	-2.32	1.49	1.23	-0.26	3.19	1.78	-1.41	60.34	51.20	-9.14
Green gram (mung dhal)	16.48	69.61	26.36	24.01	-2.35	0.70	0.65	-0.05	3.78	2.66	-1.12	52.68	46.80	-5.88
Black gram ..	15.95	58.96	25.12	22.95	-2.17	0.65	0.45	-0.20	3.47	2.29	-1.18	54.81	52.60	-2.21
Cow gram ..	12.93	63.06	24.07	22.87	-1.20	0.75	0.55	-0.20	3.29	2.19	-1.10	58.96	55.40	-3.56
Field bean, white ..	12.75	62.86	21.84	19.04	-2.80	0.85	0.78	-0.07	3.17	1.76	-1.41	61.39	53.30	-8.09
Field bean, black ..	13.26	63.23	23.41	21.66	-1.75	0.56	0.41	-0.15	3.26	2.13	-1.13	59.51	51.80	-7.71
Soya bean ..	13.30	59.71	42.41	40.59	-1.82	17.57	13.06	-4.51	3.95	2.86	-1.09	22.77	24.98	+2.21

TABLE I—*contd.*

Foodstuffs.	CALORIFIC VALUE.			CALCIUM (Ca).			PHOSPHORUS (P).			IRON (Fe).			REMARKS.
	Uncooked, per 100 g.	Cooked, per 100 g.	Deviation, per 100 g.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, mg. per cent.	Cooked, mg. per cent.	Deviation, mg. per cent.	
<i>Cereals—</i>													
Rice, raw, milled ..	338.6	290.4	-48.2	0.007	0.006	-0.001	0.188	0.079	-0.109	3.33	0.78	-2.55	Cooked in four times its weight of water; 'conjee' rejected.
Rice, parboiled, milled	335.7	293.4	-42.3	0.024	0.005	-0.019	0.239	0.087	-0.152	2.07	0.21	-2.46	Do.
Rice, raw, home-pounded.	350.8	336.0	-14.8	0.015	0.006	-0.009	0.214	0.142	-0.072	3.90	0.51	-3.39	Do.
Rice, parboiled, home-pounded.	347.3	319.0	-28.3	0.009	0.004	-0.005	0.241	0.135	-0.106	5.00	0.91	-4.09	Do.
Ragi ..	333.2	330.5	-2.7	0.410	0.405	-0.005	0.241	0.247	+0.006	15.00	11.53	-3.47	Powder cooked.
Camba ..	338.0	346.2	-11.8	0.064	0.064	0	0.311	0.313	+0.002	13.00	12.80	-0.20	Do.
Wheat ..	347.5	307.0	-40.5	0.029	0.021	-0.008	0.338	0.264	-0.074	5.00	3.34	-1.66	Broken grains cooked like rice.
Cholam (jowar) ..	354.4	347.0	-7.4	0.015	0.016	+0.001	0.309	0.314	+0.005	4.38	4.30	-0.08	Powder cooked.
<i>Pulses—</i>													
Red gram (dhal arhar)	342.4	204.0	-48.4	0.070	0.046	-0.024	0.354	0.263	-0.091	10.40	9.98	-0.42	Without outer skin.
Green gram (mung dhal)	322.5	289.0	-33.5	0.145	0.078	-0.067	0.402	0.312	-0.090	8.10	8.00	-0.10	Do.
Black gram ..	325.6	306.0	-19.6	0.320	0.073	-0.247	0.389	0.335	-0.054	8.34	2.73	-5.61	Do.
Cow gram ..	338.9	319.0	-19.9	0.076	0.060	-0.016	0.425	0.349	-0.076	5.38	4.07	-1.31	
Field bean, white ..	340.6	297.0	-43.6	0.084	0.067	-0.017	0.367	0.265	-0.102	7.70	6.02	-1.68	
Field bean, black ..	336.7	296.0	-40.7	0.062	0.062	0	0.463	0.349	-0.114	4.28	2.98	-1.30	
Soya bean ..	418.9	379.8	-39.1	0.166	0.158	-0.008	0.538	0.477	-0.061	13.85	4.67	-9.18	

TABLE I—*contd.*

Foodstuffs.	MOISTURE.		PROTEIN.			ETHER EXTRACTIVES.			MINERAL MATTER.			CARBOHYDRATES.		
	Uncooked, per cent.	Cooked, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.
<i>Leafy vegetables—</i>														
Amaranth leaves ..	89.66	90.26	2.84	2.41	-0.43	0.25	0.17	-0.08	1.57	1.42	-0.15	5.68	4.22	-1.46
Drumstick leaves ..	70.15	83.10	7.88	5.30	-2.58	1.49	1.20	-0.29	2.95	2.66	-0.29	17.53	8.71	-8.82
Cabbage ..	95.04	95.83	1.02	0.43	-0.59	0.05	0.01	-0.04	0.35	0.58	+0.23	3.54	1.77	-1.77
Spinach ..	92.51	91.03	2.97	1.99	-0.98	0.29	0.29	0	1.47	0.73	-0.74	2.76	2.24	-0.52
<i>Root and tuber vegetables—</i>														
Colocasia, tuber ..	73.65	78.91	2.64	1.88	-0.76	0.14	0.11	-0.03	0.55	0.67	+0.12	21.02	21.25	+0.23
Carrot ..	91.49	94.12	0.64	0.47	-0.17	0.10	0.06	-0.04	0.57	0.96	+0.39	7.20	4.25	-2.95
Potato ..	75.65	79.80	1.75	1.74	-0.01	0.07	0.05	-0.02	0.66	0.81	+0.15	21.87	17.70	-4.17
Sweet potato ..	63.97	73.67	1.28	0.96	-0.32	0.10	0.08	-0.02	0.70	1.67	+0.97	33.95	30.00	-3.95
Radish, white ..	91.79	94.73	0.91	0.47	-0.44	0.06	0.05	-0.01	0.68	1.45	+0.77	6.56	3.22	-3.34
Yam, elephant ..	73.87	80.65	1.23	0.91	-0.32	0.10	0.10	0	0.77	1.61	+0.84	24.03	18.62	-5.41
<i>Other vegetables—</i>														
Brinjal ..	80.39	92.53	1.24	0.99	-0.25	0.23	0.12	-0.11	0.65	0.77	+0.12	7.49	6.08	-1.41
Lady's fingers ..	89.76	93.42	2.15	1.17	-0.98	0.11	0.09	-0.02	0.84	1.29	+0.45	7.14	6.03	-1.11
Broad beans ..	90.48	91.14	2.40	1.54	-0.86	0.12	0.10	-0.02	0.59	1.23	+0.64	6.41	5.49	-0.92
Cluster beans ..	86.62	87.50	3.02	1.90	-1.12	0.08	0.05	-0.03	1.00	1.84	+0.84	9.28	7.32	-1.96
Drumstick ..	89.52	88.22	3.79	1.94	-1.85	0.44	0.12	-0.32	0.99	1.13	+0.14	5.26	7.43	+2.17
French beans ..	86.72	86.92	1.89	1.30	-0.59	0.09	0.07	-0.02	0.69	1.37	+0.68	10.61	10.60	-0.01
Plantain, green ..	71.94	76.61	1.12	0.84	-0.28	0.16	0.08	-0.08	0.74	1.64	+0.90	26.04	28.20	+2.16
Ridge-round ..	94.97	93.13	0.54	0.58	+0.04	0.09	0.05	-0.04	0.27	0.67	+0.40	4.13	3.92	-0.21

TABLE I—*concl'd.*

Foodstuffs.	CALORIFIC VALUE.			CALCIUM (Ca).			PHOSPHORUS (P).			IRON (Fe).			REMARKS.	
	Uncooked, per 100 g.	Cooked, per 100 g.	Deviation, per 100 g.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, per cent.	Cooked, per cent.	Deviation, per cent.	Uncooked, mg. per cent.	Cooked, mg. per cent.	Deviation, mg. per cent.		
<i>Leafy vegetables—</i>													Cooked with common salt.	
Amaranth leaves ..	36.3	28.1	— 8.2	0.153	0.167	+0.014	0.045	0.021	—0.024	13.90	16.60	+2.70		Do.
Drumstick leaves ..	115.1	67.0	—48.1	0.648	0.405	—0.153	0.008	0.036	—0.002	6.18	5.73	—0.45		Do.
Cabbage ..	18.7	8.9	— 9.8	0.038	0.018	—0.020	0.021	0.009	—0.012	0.71	1.14	+0.40		Do.
Spinach ..	25.5	19.5	— 6.0	0.076	0.072	—0.004	0.026	0.013	—0.013	7.96	5.67	—2.29		Do.
<i>Root and tuber vegetables—</i>														
Colocasia, tuber ..	95.9	93.5	— 2.4	0.023	0.027	+0.004	0.035	0.131	+0.009	0.78	1.16	+0.38	Do.	
Carrot ..	30.3	19.4	—10.9	0.023	0.021	—0.002	0.025	0.015	—0.010	0.79	1.17	+0.38	Do.	
Potato ..	95.1	78.2	—16.9	0.063	0.065	+0.002	0.037	0.036	—0.001	0.63	0.48	—0.15	Do.	
Sweet potato ..	141.8	124.5	—17.3	0.023	0.022	—0.001	0.011	0.021	—0.020	0.88	0.92	+0.04	Do.	
Radish, white ..	30.4	15.2	—15.2	0.020	0.030	+0.001	0.029	0.014	—0.015	0.70	0.88	+0.18	Do.	
Yam, elephant ..	101.9	79.0	—22.9	0.085	0.086	+0.001	0.024	0.020	—0.004	0.57	1.37	+0.80	Do.	
<i>Other vegetables—</i>														
Brinjal ..	37.0	29.4	— 7.6	0.019	0.016	—0.003	0.046	0.028	—0.018	0.85	1.12	+0.27	Do.	
Lady's fingers ..	38.2	29.6	— 8.6	0.090	0.078	—0.012	0.058	0.038	—0.020	1.50	1.25	—0.25	Do.	
Broad beans ..	36.3	29.0	— 7.3	0.032	0.025	—0.007	0.051	0.026	—0.025	1.25	1.39	+0.14	Do.	
Cluster beans ..	49.9	37.3	—12.6	0.106	0.068	—0.038	0.059	0.031	—0.028	2.17	2.11	—0.06	Do.	
Drumstick ..	40.2	38.6	— 1.6	0.030	0.027	—0.003	0.065	0.044	—0.021	0.86	1.22	+0.36	Do.	
French beans ..	50.8	48.6	— 2.2	0.045	0.032	—0.013	0.064	0.041	—0.023	1.67	1.82	—0.05	Do.	
Plantain, green ..	110.1	117.0	+ 6.9	0.010	0.014	+0.004	0.029	0.019	—0.010	0.67	0.84	+0.17	Do.	
Ridge-gourd ..	19.5	18.4	— 1.1	0.010	0.015	+0.005	0.058	0.037	—0.021	0.58	0.95	+0.37	Do.	
Snake-gourd ..	18.2	12.6	— 5.6	0.020	0.019	—0.001	0.018	0.010	—0.008	0.55	1.94	+1.39	Do.	

TABLE II—*concl'd.*

	Moisture, per cent.	Protein, per cent.	Ether extract- ives, per cent.	Mineral matter, per cent.	Carbo- hydrates, per cent.	Calorific value, per 100 g.	Calcium (Ca), per cent.	Phospho- rus (P), per cent.	Iron (Fe), mg. per cent.
10. Rice, parboiled, milled, cooked with seven times its weight of water.	78.89	5.70	0.20	0.30	76.60	332.0	0.003	0.067	0.67
11. Rice, parboiled, milled, cooked with 10 times its weight of water.	76.15	5.30	0.09	0.23	69.10	298.0	0.002	0.051	0.62
12. 'Conjee' from 9 ..	96.18	0.19	0.004	0.13	6.40	26.5	0.0003	0.023	0.26
13. 'Conjee' from 10 ..	97.79	0.18	0.02	0.18	7.78	32.2	0.0005	0.030	0.27
14. 'Conjee' from 11 ..	98.17	0.33	0.02	0.25	11.82	49.0	0.0008	0.043	0.43
15. Rice, raw, home-pounded ..	12.69	7.86	1.29	1.22	76.94	350.8	0.015	0.214	3.90
16. Rice, raw, home-pounded, cooked with four times its weight of water.	77.59	7.68	0.67	0.56	74.90	336.0	0.006	0.142	0.51
17. Rice, raw, home-pounded, cooked with seven times its weight of water.	81.93	7.47	0.58	0.46	69.30	312.0	0.004	0.102	0.62
18. Rice, raw, home-pounded, cooked with 10 times its weight of water.	79.25	7.54	0.48	0.42	71.70	321.0	0.004	0.074	0.56

19. 'Conjee' from 16	..	95.66	0.19	0.06	0.06	3.84	10.2	0.0004	0.009	0.09
20. 'Conjee' from 17	..	98.26	0.21	0.04	0.11	5.39	22.2	0.0009	0.017	0.18
21. 'Conjee' from 18	..	98.81	0.19	0.03	0.15	7.10	29.8	0.0010	0.022	0.50
22. Rice, parboiled, home-pounded		12.50	7.66	0.53	1.34	77.97	347.3	0.009	0.244	5.09
23. Rice, parboiled, home-pounded, cooked with four times its weight of water.		80.52	7.40	0.44	0.56	71.10	319.0	0.004	0.135	0.01
		74.56	7.35	0.44	0.50	69.20	310.0	0.004	0.112	0.87
		77.77	7.17	0.35	0.44	69.00	308.0	0.002	0.108	1.03

<i>Raw home-pounded rice—</i>										
7.	'Conjee' from raw home-pounded rice, cooked in four times its weight of water.	95.66	0.20	0.006	0.061	4.07	17.2	0.0005	0.0100	0.037
8.	Same as 7, but cooked in seven times its weight of water.	98.26	0.06	0.003	0.033	1.65	6.8	0.0003	0.0052	0.086
9.	Same as 7, but cooked in 10 times its weight of water.	98.81	0.04	0.001	0.023	1.12	4.7	0.0002	0.0034	0.079
<i>Parboiled home-pounded rice—</i>										
10.	'Conjee' from parboiled home-pounded rice, cooked in four times its weight of water.	95.63	0.07	0.010	0.069	4.22	17.3	0.0010	0.0111	0.390
11.	Same as 10, but cooked in seven times its weight of water.	98.81	0.06	0.003	0.052	1.08	4.6	0.0002	0.0083	0.010
12.	Same as 10, but cooked in 10 times its weight of water.	99.02	0.04	0.002	0.040	0.90	3.8	0.0001	0.0067	0.005

TABLE I—concl'd.

Name of foodstuff.	Botanical name.	Moisture, per cent.	'Crude protein' ($N \times 6.25$), per cent.
Ragi	<i>Eleusine coracana</i>	12.68	7.43
Red gram	<i>Cajanus indicus</i>	13.08	23.57
Lentil	<i>Lens esculenta</i>	12.46	24.11
Bengal gram ..	<i>Cicer arietinum</i>	12.94	21.68
Green gram ..	<i>Phaseolus radiatus</i>	13.10	24.77
Black gram ..	<i>Phaseolus mungo</i>	12.82	24.23
Soya bean	<i>Glycine hispida</i>	10.25	42.41
Skimmed milk powder ..	—	4.10	38.04

The composition of the diets used is shown in Table II. They are similarly constituted to those used in Part I of this series of experiments (Swaminathan, *loc. cit.*).

EXPERIMENTAL.

Groups of young rats (3 or 4 in each group), of 45 g. to 55 g. body-weight, were fed individually with the various experimental diets, in the proportions indicated in Table II. In addition 3 c.c. of an aqueous solution of yeast extract (corresponding to 0.75 g. of dry yeast), containing the vitamin-B complex, were given daily to each rat along with its food. The diets were thoroughly mixed up, made into a paste by adding distilled water in order to prevent scattering of food, and samples were analysed for their moisture and nitrogen content. The dry weights of the diets before the addition of water (allowing for the moisture contents of the foodstuffs used) were noted and also their wet weights in the pasty condition. In weighing out the daily food care was taken that the food given should not exceed greatly the average food consumption, thus avoiding a large residue. Food residue remaining uneaten was carefully collected daily, completely dried in an air oven at 100°C., and the corresponding dry weights of the residual food subtracted from the dry weight of the food given daily to the rats. By this means daily food intake was obtained.

The test diets were fed for a period of 9 weeks. The first week of each period was regarded as preparatory, allowing the rats to accommodate themselves to the diets; the daily collection of food residue was therefore confined to the remaining 8 weeks of the period and calculations were made on the results of the subsequent 8 weeks. The rats were also weighed weekly.

The data regarding the growth experiments and calculation of the biological values are given in Table III.

TABLE III—*contd.*
Biological values of the proteins of ragi and lentil.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein (N \times 6.25) in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
14 M 15 F	28	Ragi 5.07	49 54	59 63	10 9	200.8 191.0	10.18 9.68	0.98 0.93	0.96
14 M 15 F	56	Ragi 5.07	49 54	65 68	16 14	415.0 415.9	21.04 21.09	0.76 0.66	0.71
19 M 20 F 21 F 22 M	28	Lentil 10.13	48 55 52 51	67 68 65 68	19 13 13 17	283.5 247.2 255.6 248.2	28.72 25.04 25.89 25.14	0.66 0.52 0.50 0.68	0.59
19 M 20 F 21 F 22 M	56	Lentil 10.13	48 55 52 51	80 76 75 76	32 21 23 25	617.4 488.0 489.5 515.2	62.54 49.44 49.59 52.19	0.51 0.43 0.46 0.48	0.47

TABLE III—*contd.*
Biological values of the proteins of black gram and soya bean.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein (N x 0.25) in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
31 M	28	Black gram 10-67	63	90	33	237.6	25.35	1.30	1.29
32 M			65	105	40	276.4	29.46	1.36	
33 M			69	104	35	258.9	27.03	1.28	
34 F			58	92	34	264.9	27.84	1.22	
31 M	56	Black gram 10-67	63	120	57	528.4	56.35	1.01	1.00
32 M			65	135	70	600.0	64.02	1.03	
33 M			69	125	56	560.5	59.84	0.94	
34 F			58	120	62	565.3	60.32	1.11	
35 M	28	Soya bean 10-34	54	82	28	277.6	28.70	0.98	0.94
36 M			62	96	24	280.7	29.03	0.83	
37 M			62	90	28	284.6	29.43	0.95	
38 F			67	95	28	268.5	27.76	1.01	
35 M	56	Soya bean 10-34	54	100	46	578.9	59.86	0.77	0.78
36 M			62	102	40	575.2	59.48	0.67	
37 M			62	110	48	569.9	58.93	0.82	
38 F			67	114	47	519.8	53.75	0.87	

DISCUSSION.

The problem of measuring the nutritive value of proteins for growth is complicated by the fact that the protein requirement for growth is superimposed upon that for maintenance. Hence, in simple feeding experiments, the growth secured is not the total effect of the dietary protein consumed, since part of the protein is being used to replace endogenous losses of nitrogen (maintenance). The questions as to whether the protein requirements for maintenance are the same as requirements for growth, either qualitatively or quantitatively, and whether it is possible to arrive at the protein requirements for growth by subtracting the protein the requirements for maintenance, observed in experiments in which both are involved, will be discussed later, in the light of the existing literature and the results obtained in these investigations. In comparing the biological values of proteins by this method, it should also be remembered that the digestibility of the proteins themselves is also an influencing factor. It was pointed out in the introduction that results of experiments of varying length are not comparable, since short experiments give higher values than longer ones. The duration of the experimental period in this study was 8 weeks, and the calculation of the biological value has been made from the data obtained for the first period of 4 weeks and for 8 weeks. In general the figures obtained during a period of 4 weeks were slightly higher than the corresponding values obtained during a period of 8 weeks. This has also been the experience of previous workers (Osborne and Mendel, 1920; Basu *et al.*, *loc. cit.*). The level of protein intake was kept at approximately 5 per cent in the diets containing the cereals and at 10 per cent in those containing pulses, since the protein content of the former varied from 6 to 12 per cent and it is not possible to prepare diets containing 10 per cent protein with all the cereals. Hence comparisons cannot be made between cereals and pulses, since the levels of protein intake are not the same.

Cereals.

Of the 5 cereals—rice, wheat, cambu, cholam, and ragi—investigated, the proteins of rice have the highest biological value, 2.17 and 1.7, during periods of 4 weeks and 8 weeks respectively. Morgan (1931) reported a value of 1.41 for the proteins of rice when the duration of experimental period was 8 weeks and the level of protein intake 8 per cent. The high biological value (80) obtained for the proteins of rice by the balance-sheet method for maintenance in adult rats, together with the high value (1.7 during 8 weeks) obtained for the maintenance and growth of young rats, go to show that the proteins of rice are of high quality for supporting maintenance and growth. The proteins of wheat rank next to those of rice, the biological values obtained being 1.60 and 1.31 during periods of 4 weeks and 8 weeks respectively. Osborne and Mendel (1920) reported values of 1.2 and 1.1 during the periods of 4 weeks and 10 weeks, when the level of protein intake was 5 per cent and the source of vitamin-B complex was wheat itself. The few other recorded estimations of the biological values of the proteins of wheat using this method are as follows: 1.6 during a period of 60 days and at a level of intake of protein of 10 per cent (Hoagland and Snider, 1926); 1.7 during a period of 56 days (8 weeks) and at a level of protein intake of 10 per cent (Morgan, *loc. cit.*); and 1.36 during a period of 9 weeks and at a level of protein intake of 10 per cent

(Boas-Fixsen *et al.*, 1934). The biological value of 66 obtained for the proteins of wheat by the balance-sheet method for maintenance in adult rats, together with the values of 1·60 and 1·31 obtained for the growth and maintenance of young rats, go to prove that the proteins of wheat are inferior to those of rice.

The proteins of cambu, cholam, and ragi are of lower value for the growth and maintenance of young rats, than those of rice and wheat, ranking in the following descending order—cambu, cholam, and ragi. The biological values obtained are 1·27 and 1·15 for the proteins of cambu, 0·87 and 0·78 for those of cholam and 0·96 and 0·71 for those of ragi, during the periods of 4 weeks and 8 weeks respectively. On the other hand, high biological values—83, 83, and 89 respectively—were obtained for the proteins of these foodstuffs for maintenance in adult rats (Swaminathan, *loc. cit.*). From the above results, it is evident that the proteins of these three foodstuffs are more efficient for maintenance than for growth. The cereal proteins may be arranged in the following descending order of merit for the growth and maintenance of young rats: rice, wheat, cambu, cholam, and ragi.

Pulses.

Of the 6 pulses—red gram, lentil, Bengal gram, green gram, black gram, and soya bean—investigated, the proteins of black gram were found to be the best and those of red gram the poorest. While biological values of 1·29 and 1·00 were obtained for the proteins of black gram, the proteins of red gram were found to support no growth at all. All the three rats placed on the red-gram diet failed to grow and died during the course of the experiment. In later experiments a fairly high biological value (62) has been obtained for the proteins of black gram for maintenance in adult rats. This figure, and the values (1·29 and 1·00) obtained here, show that black gram contains proteins of fairly high value. The proteins of green gram rank next to those of black gram, the biological values obtained being 1·29 and 0·94 during the periods of 4 and 8 weeks respectively, Basu *et al.* (*loc. cit.*) reporting slightly higher values of 1·48 and 1·16 respectively.

The proteins of soya bean have been the subject of numerous investigations, but the available data is still conflicting. Osborne and Mendel (1916), Shrewsbury and Vestel (1932), and Shrewsbury *et al.* (1932) found that raw soya beans when fed to rats as the sole or principal source of protein in an otherwise adequate diet did not support normal growth. However normal growth resulted when they fed soya bean which had been previously cooked. Kon and Markuze (*loc. cit.*) obtained a value of 1·57 for the proteins of steam-cooked soya bean during a period of 30 days when fed at 10 per cent level. Adolph and Cheng (1935) reported a biological value of 1·4 for the proteins of soya bean during a period of 30 days when fed at 11·5 per cent level. The recent findings of Steenbock *et al.* (1936) are not in agreement with those of the previous investigators. They found that raw soya beans produced no growth when the level of protein intake was 10 per cent and only a slight growth at an 18 per cent level, the biological value obtained at the higher level being 0·5. In the present experiments, it was found that the proteins of raw soya beans produced moderate growth, the biological values obtained being 0·94 and 0·78 during periods of 4 and 8 weeks respectively, a result not in agreement with those of the previous investigators. The higher

value reported by Kon and Markuze (*loc. cit.*) may be due to the effect of cooking on the proteins of soya bean, and that reported by Adolph and Cheng (*loc. cit.*) to the supplementary effect of the proteins of dried yeast used in their experiments as the source of the vitamin-B complex.

The proteins of lentil were found to support very poor growth, the biological values obtained being 0.59 and 0.47 during periods of 4 and 8 weeks. Basu *et al.* (*loc. cit.*) obtained higher values of 1.05 and 0.57.

Skimmed milk powder.

Biological values of 1.84 and 1.45 were obtained for the proteins of skimmed milk powder, during periods of 4 and 8 weeks respectively and at a 10 per cent level of protein intake. These values are nearly twice that obtained for the proteins of soya bean under similar conditions. Hoagland and Snider (*loc. cit.*) reported a higher biological value of 2.36 for the proteins of milk, during a period of 30 days and at a 10 per cent level of protein intake.

FOOD INTAKE AND INCREASE IN BODY-WEIGHT.

Osborne and Mendel (1915) have shown that rats consumed food in proportion to their calorie needs. The diets shown in Table II are of almost equal calorific value per unit of weight. From Table IV it will be seen that the average daily food intake with the diets containing the cereals, during the period of 8 weeks, varies from 6.8 g. with the rice diet to 10.3 g. with the cholam diet; with the diets containing the pulses it varied from 8.1 g. with the green-gram diet to 10.1 g. with the black-gram diet. The daily average food intake of rats on skimmed milk powder diet was 11.7 g. Mitchell and Hamilton (*loc. cit.*) are of opinion that 'the greater the intake of food by rats of similar size, the greater the proportion of protein intake that will be available for growth and hence the greater the gain per gram of protein consumed'. The results obtained in the experiments do not wholly support this contention, for although greater food intake is associated in general with greater increase in body-weight, the ratio $\frac{\text{g. gain in body-weight}}{\text{g. protein intake}}$ is only slightly affected, since greater protein intake is followed by a corresponding greater increase in weight.

EFFECT OF SEX ON THE BIOLOGICAL VALUE OF PROTEINS.

Hoagland and Snider (*loc. cit.*) reported that, throughout a large number of comparisons, the gain in weight per gramme of protein intake was, with two exceptions, larger in male rats than in female rats, and they interpreted this difference as being the result of the greater growth capacity of male rats as compared with female rats resulting in a greater consumption of food. The experiments of Morgan (*loc. cit.*), on the other hand, do not wholly support this hypothesis. He found that although greater gains in weight are usually made by the males their food intake is also greater and the factor gain per gramme of protein intake is but little affected. Basu *et al.* (*loc. cit.*) found that with a single exception male rats gave slightly higher values than female ones. In

TABLE IV.

Average total, weekly, and daily food intake, protein intake, and gain in body-weight with diets containing cereals.

Source of protein in the diet.	Percentage of protein (N \times 6.25).	Duration of experimental period, days.	AVERAGE FOOD INTAKE.			AVERAGE PROTEIN INTAKE.			AVERAGE INCREASE IN BODY-WEIGHT.		
			Total.	Weekly.		Total.	Weekly.		Total.	Weekly.	Daily.
Raj milled rice	5.22	28	166.2	41.6	5.9	8.67	2.17	0.31	19	4.8	0.69
	5.22	56	382.0	47.8	6.8	19.94	2.49	0.36	34	4.3	0.61
Whole wheat	5.36	28	215.3	53.8	7.7	11.54	2.89	0.41	19	4.8	0.69
	5.36	56	459.9	57.5	8.2	24.65	3.08	0.44	32	4.0	0.57
Cambu	5.45	28	248.8	62.2	8.9	13.56	3.39	0.48	17	4.3	0.61
	5.45	56	493.7	61.7	8.8	26.92	3.37	0.48	31	3.9	0.56
Cholam	5.17	28	293.7	73.4	10.5	15.18	3.80	0.54	13	3.3	0.47
	5.17	56	579.4	72.4	10.3	29.99	3.62	0.52	24	3.0	0.43
Ragi	5.07	28	195.9	49.0	7.0	9.93	2.48	0.35	10	2.4	0.34
	5.07	56	415.5	51.9	7.4	21.07	2.63	0.38	15	1.9	0.27

TABLE IV—*concl'd.*

Average total, weekly, and daily food intake, protein intake and gain in body-weight with diets containing pulses and skimmed milk powder.

Source of protein in the diet.	Percentage of protein (N \times 6.25).	Duration of experimental period, days.	AVERAGE FOOD INTAKE.			AVERAGE PROTEIN INTAKE.			AVERAGE INCREASE IN BODY-WEIGHT.		
			Total.	Weekly.	Daily.	Total.	Weekly.	Daily.	Total.	Weekly.	Daily.
Lentil	10.13	28	258.6	64.7	9.2	26.20	6.55	0.93	16	4.0	0.57
	10.13	56	527.5	65.9	9.4	53.44	6.68	0.95	25	3.1	0.44
Bengal gram	10.22	28	237.1	59.3	8.5	24.23	6.06	0.87	23	5.8	0.83
	10.22	56	514.2	64.3	9.2	52.56	6.57	0.94	38	4.8	0.69
Green gram	10.19	28	203.8	51.0	7.3	20.76	5.19	0.74	26	6.5	0.93
	10.19	56	452.9	56.6	8.1	46.18	5.77	0.82	43	5.4	0.77
Black gram	10.67	28	258.4	64.6	9.2	27.57	6.89	0.98	36	9.0	1.29
	10.67	56	563.6	70.5	10.1	60.13	7.52	1.07	61	7.6	1.09
Soya bean	10.34	28	277.9	69.5	9.9	28.73	7.18	1.03	27	6.8	0.97
	10.34	56	561.0	70.1	10.0	58.00	7.25	1.04	45	5.6	0.80
Skimmed milk powder.	10.37	28	306.1	76.5	10.9	31.74	7.94	1.12	59	14.8	2.11
	10.37	56	654.6	81.8	11.7	67.88	8.49	1.21	98	12.3	1.76

the present experiments, with two exceptions, the values obtained for male rats are slightly in excess of those obtained with female rats. The biological values obtained with male and female rats are shown in Table V:—

TABLE V.

Effect of sex on the biological values.

Source of protein in the diet.	Approximate level of protein in the diet, per cent.	Number of rats.	AVERAGE BIOLOGICAL VALUE.	
			4 weeks.	8 weeks.
Raw milled rice ..	5	2 M	2.27	1.86
		1 F	1.98	1.38
Whole wheat ..	5	2 M	1.77	1.43
		2 F	1.43	1.18
Cambu	5	2 M	1.37	1.27
		1 F	1.08	0.92
Cholam	5	2 M	0.96	0.90
		1 F	0.69	0.53
Ragi	5	1 M	0.98	0.76
		1 F	0.93	0.66
Lentil	10	2 M	0.67	0.50
		2 F	0.51	0.45
Bengal gram ..	10	2 M	1.04	0.75
		2 F	0.78	0.65
Green gram ..	10	2 M	1.31	0.99
		2 F	1.27	0.90

TABLE V—concl'd.

Source of protein in the diet.	Approximate level of protein in the diet, per cent.	Number of rats.	AVERAGE BIOLOGICAL VALUE.	
			4 weeks.	8 weeks.
Black gram ..	10	3 M	1.31	0.99
		1 F	1.22	1.03
Soya bean ..	10	3 M	0.92	0.75
		1 F	1.01	0.87
Skimmed milk powder	10	3 M	1.92	1.48
		1 F	1.61	1.38

The biological values and the values of $\frac{I^2}{TP}$.

Osborne, Mendel and Ferry's method relates the growth obtained (I) to the protein intake (P) and expresses the biological value of the protein by the ratio $\frac{\text{g. gain in body-weight } (I)}{\text{g. protein intake } (P)}$. On the other hand Plimmer *et al.* (*loc. cit.*) pointed out that increase in body-weight (I), the time or duration of experiment (T) and the protein intake (P) together represent the value of a protein in nutrition, i.e., $\frac{I}{T}$ is the first factor representing the growth curve and $\frac{I}{P}$ is the second factor representing the gain in weight per gramme of protein intake. According to them the product of the two factors $\frac{I}{T}$ and $\frac{I}{P}$, i.e., $\frac{I^2}{TP}$, represents completely the nutritive value of the protein numerically. The average values of the factor $\frac{I^2}{TP}$ have been calculated for periods of 28 days and 56 days and are shown in Table VI. In these calculations, $\frac{I}{T}$ is taken as the average daily increase in body-weight. It will be seen from Table VI that the values of $\frac{I^2}{TP}$ are less than the corresponding biological values except in the cases of black gram and skimmed milk powder where they are greater, due to the obvious reason that with these foods the average daily increase in body-weight exceeded one gramme. Nevertheless, the values of $\frac{I^2}{TP}$ provide roughly similar conclusions as regards the relative biological value of proteins investigated.

TABLE VI.

Showing average biological values and the values of $\frac{I^2}{TP}$.

Name of foodstuff.	Approximate level of protein, per cent.	AVERAGE BIOLOGICAL $\left(\frac{I}{P}\right)$ VALUE.		AVERAGE DAILY INCREASE IN $\frac{I}{T}$ IN BODY-WEIGHT.		AVERAGE VALUE $\frac{I}{P} \times \frac{I}{T}$, i.e., $\frac{I^2}{TP}$, OF	
		28 days.	56 days.	28 days.	56 days.	28 days.	56 days.
Raw milled rice ..	5	2.17	1.70	0.69	0.61	1.50	1.04
Whole wheat ..	5	1.60	1.31	0.66	0.57	1.06	0.75
Cambu ..	5	1.27	1.15	0.61	0.56	0.77	0.64
Cholam ..	5	0.87	0.78	0.47	0.43	0.41	0.34
Ragi ..	5	0.96	0.71	0.34	0.27	0.33	0.19
Lentil ..	10	0.59	0.47	0.57	0.44	0.34	0.21
Bengal gram ..	10	0.91	0.70	0.83	0.69	0.76	0.48
Green gram ..	10	1.29	0.94	0.93	0.77	1.20	0.72
Black gram ..	10	1.29	1.00	1.29	1.09	1.66	1.09
Soya bean ..	10	0.94	0.78	0.97	0.80	0.91	0.62
Skimmed milk powder ..	10	1.84	1.45	2.11	1.76	3.88	2.55

AVERAGE DAILY GAIN IN BODY-WEIGHT AND BIOLOGICAL VALUE.

McCollum *et al.* (*loc. cit.*) carried out a series of experiments with diets containing equal proportions of different proteins. The performances of the rats fed on these diets were compared not only as regards rate of growth but also as regards longevity, fertility, and lactation, no records being made of the food intake of rats. Mitchell (1929*b*) has shown from data based on the experiments

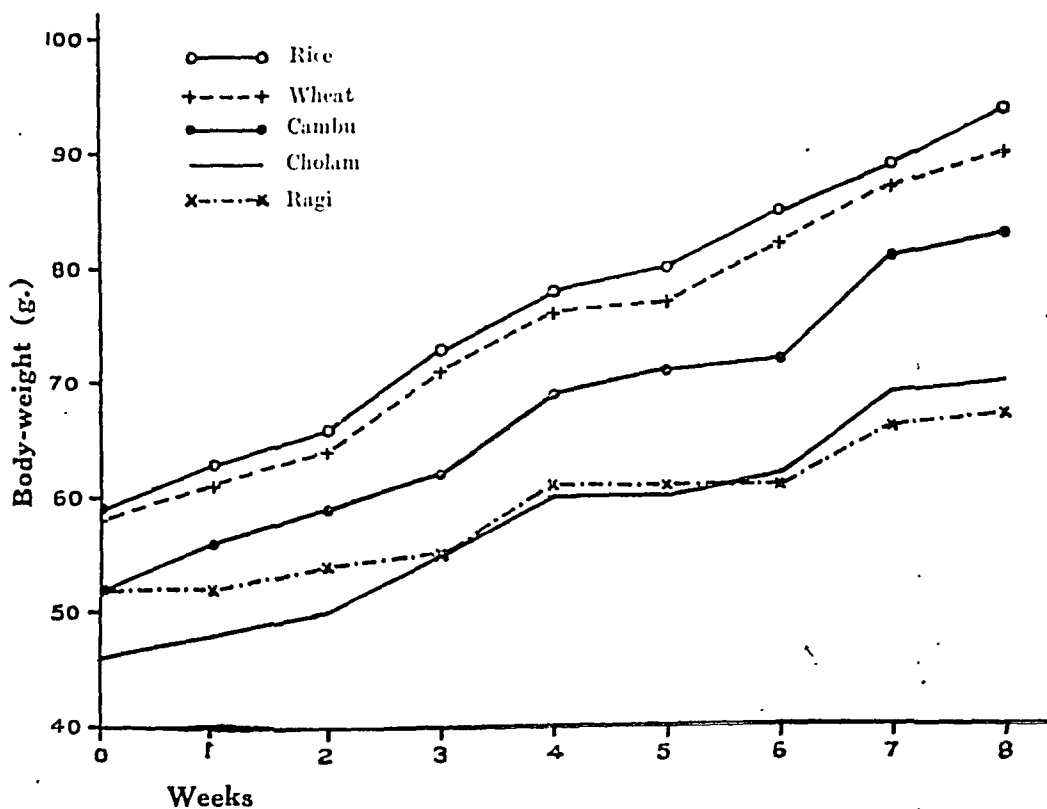


Fig. 1.—Increase in body-weight in rats obtaining proteins from various cereals.

of Osborne and Mendel (1920) that results based only upon the rate of growth of young rats, without taking into consideration the food and protein intake, lead to erroneous conclusions about the relative biological value of different proteins. Taking average daily gain in body-weight as the sole criterion of biological value (McCollum *et al.*, *loc. cit.*) the proteins investigated in these experiments would range themselves as follows in descending order of merit: cereal proteins—rice, wheat,

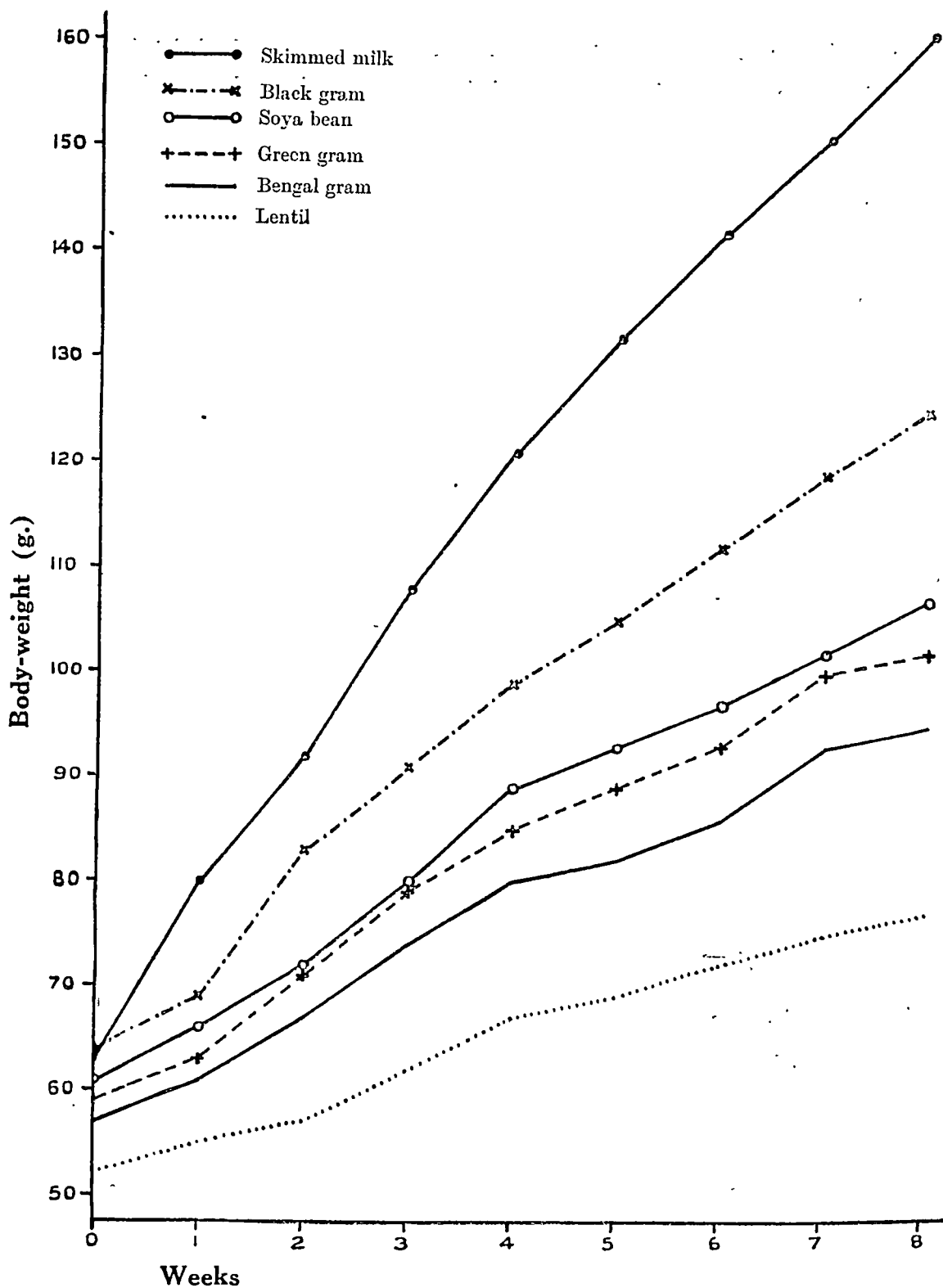


Fig. 2.—Increase in body-weight in rats obtaining proteins from various pulses and skimmed milk powder.

cambu, cholam, and ragi; pulse proteins: black gram, soya bean, green gram, Bengal gram, and lentil. The conclusions so arrived at are in general agreement with the biological values assessed by the standard growth method and the values of $\frac{I^2}{TP}$. A single exception is that the proteins of soya bean are placed above those of green gram. The average daily increase of body-weight is given in Table VI along with the corresponding biological values and the values of $\frac{I^2}{TP}$; the growth curves are shown in Figs. 1 and 2.

THE BIOLOGICAL VALUES OF PROTEINS FOR DIFFERENT FUNCTIONS: MAINTENANCE VERSUS GROWTH.

It seems unlikely that the protein requirements for maintenance should be the same either quantitatively or qualitatively as those for growth. Part of the first will be used to form simple nitrogenous compounds, whereas for the second a complex selection of all the amino acids which enter into the structure of tissue proteins will be needed. It has been repeatedly demonstrated by many workers (Osborne and Mendel, 1911; Willcock and Hopkins, 1906-07; Rose and Cox, 1924, 1926) that the differences in nutritive value for growth and maintenance displayed by different proteins are mainly due to the presence in varying proportions, or the absence, of certain essential amino acids such as tryptophane, lysine, cystine, and histidine. Since amino-acid requirements for growth and maintenance differ, it is impossible to assess accurately the value of a protein for growth alone by subtracting the amount estimated as necessary for maintenance in experiments in which both growth and maintenance were involved. In this connection, Sherman (1933) observes that 'It may also be helpful to think of the protein metabolism, not only in terms of building and repair, but also of maintaining the (approximate) dynamic equilibrium which exists between proteins and amino acids in the cells of animal tissues. Concentration of any one of the amino acids into which tissue proteins tend to be hydrolysed may therefore be expected to help in pushing the reaction amino acids \rightleftharpoons proteins towards the right; in other words any of these amino acids may thus, to some extent, function in the maintenance of body protein, whereas for the synthesis of new protein as in growth, all the amino acids which enter into the structure of tissue proteins would be needed. Hence it is quite reasonable that proteins of different efficiency for growth may show much more nearly equal efficiency in the normal maintenance nutrition of adults; though it is also true that, so far as is known, the proteins more efficient for growth are likewise more efficient for maintenance'.

The results obtained in the present series of investigations which are shown in Table VII are in accordance with the view expressed by Sherman, viz., that proteins more efficient for growth are likewise more efficient for maintenance, the important examples being the proteins of milk, rice, wheat, and black gram. At the same time there is some evidence to show that proteins inefficient for growth in young rats may be very efficient for maintenance in adult rats when studied by the balance-sheet method, examples being the proteins of ragi, red gram, and cholam.

TABLE VII.

Biological values for the maintenance of adult rats and for the growth and maintenance of young rats.

Name of foodstuff.	Level of protein intake, per cent.	BIOLOGICAL VALUE.	
		For maintenance in adult rats, per cent.	For growth and maintenance in young rats during 8 weeks.
Raw milled rice ..	5	80	1.70
Whole wheat ..	5	66	1.31
Cambu	5	83	1.15
Cholam	5	83	0.78
Ragi	5	89	0.71
Red gram	10	85	—
Lentil	10	41	0.47
Bengal gram ..	10	62	0.70
Green gram ..	10	51	0.94
Black gram ..	10	62	1.00
Soya bean	10	54	0.78
Skimmed milk powder ..	10	83	1.45

SUMMARY AND CONCLUSIONS.

(1) The biological values of the proteins of 12 common foodstuffs, including 5 cereals—rice, wheat, cambu, cholam, and ragi; 6 pulses—red gram, lentil, Bengal gram, black gram, green gram, and soya bean; and skimmed milk powder, have been determined by the growth method on rats.

(2) On a 5 per cent level of protein intake, the biological values of the proteins of cereals, during periods of 4 and 8 weeks respectively, were as follows: rice 2.17 and 1.70; wheat 1.60 and 1.31; cambu 1.27 and 1.15; cholam 0.87 and 0.78; and ragi 0.96 and 0.71.

(3) On a 10 per cent level of protein intake, the biological values of the proteins of pulses and skimmed milk powder, observed during periods of 4 and 8 weeks, were as follows: lentil 0.59 and 0.47; Bengal gram 0.91 and 0.78; green gram 1.29 and 0.94; black gram 1.29 and 1.00; soya bean 0.94 and 0.78; and skimmed milk powder 1.84 and 1.45.

(4) The proteins of the five cereals investigated range themselves in the following descending order of merit for the growth and maintenance of young rats: rice, wheat, cambu, cholam, and ragi: those of the pulses as follows: black gram, green gram, soya bean, Bengal gram, and lentil.

(5) The proteins of red gram were found to be inadequate even for supporting life in young rats. On the other hand the proteins of skimmed milk powder were found to support very good growth in young rats (12 g weekly) more than twice the average weekly increase in body-weight (5.6 g) obtained with the diet containing the same percentage of protein from soya bean.

(6) The values of I_p^{12} were calculated for all the proteins investigated for periods of 1 weeks and 8 weeks. They are in agreement with the corresponding biological values obtained by the growth method.

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from the British Drug Houses, Ltd., London; it was not chemically pure and hence its content of adrenaline was estimated at every stage.

The buffers used ranged from almost 1 to 13, made up of glycocoll and either hydrochloric acid or sodium hydroxide, and of mixtures of primary and secondary phosphates, prepared according to the tables given by Clark (1920). Varying amounts of current passed through the adrenaline solution, depending on the conductivity of the buffer. To make comparison of the results possible, the amounts of adrenaline migrating to either pole are expressed on an uniform basis of 100 milliampere-hours. (Current strength in milliamperes \times duration of passage of current in hours.) The results are shown in the Table:—

TABLE.
The migration of adrenaline at various levels of pH.

pH.	Amount in mg. migrating to +ve chamber per 100 m. a. h.	Amount in mg. migrating to -ve chamber per 100 m. a. h.	ADRENALINE MIGRATING, CALCULATED AS A PERCENTAGE OF THE TOTAL LEFT AT THE END OF EACH EXPERIMENT IN CENTRAL CHAMBER.	
			+ve chamber.	-ve chamber.
1.146	<i>Nil</i>	0.109	<i>Nil</i>	14.0
1.251	<i>Nil</i>	0.192	<i>Nil</i>	33.5
3.341	<i>Nil</i>	0.319	<i>Nil</i>	42.0
7.381	<i>Nil</i>	0.406	<i>Nil</i>	71.0
7.731	Faint trace	0.310	Faint trace	50.2
8.043	Trace	0.095	Trace	27.5
8.575	0.013	0.016	2.4	2.9
8.929	0.026	0.017	4.2	2.7
9.714	0.047	<i>Nil</i>	18.1	<i>Nil</i>
10.140	0.091	<i>Nil</i>	34.9	<i>Nil</i>
12.399	0.049	<i>Nil</i>	49.0	<i>Nil</i>

It is seen from the Table that adrenaline migrates to the electro-negative pole in acid and slightly alkaline ranges, and to the electro-positive pole in strong alkaline ranges. The last two columns, showing the amount of adrenaline migrating, calculated as a percentage of the total left behind at the end of each experiment, bring out the results clearly: appreciable migration to the electro-negative pole up to pH 8.0, increasing to begin with and then steadily declining as the pH

approaches the iso-electric point, and a progressive increase in migration to the electro-positive pole with increase of pH in alkaline range. The iso-electric point, the point at which there is the least migration to either pole, is in the neighbourhood of 8.5.

The finding of Devrient *et al.*, that adrenaline migrates unequally in the direction of both poles, may possibly be explained by the fact that their results represent the summation of the effects of electrical migration and mechanical diffusion. Other observations made by them, to the effect that a greater amount of adrenaline migrates to the positive pole with the addition of alkali or alkaline salts, and to the opposite pole with the addition of acid or acid salts, are fully confirmed.

The specimen of adrenaline obtained from the B. D. H. was not chemically pure. In order to ascertain that the substance actually migrating under the influence of an electrical field was adrenaline and not any other impurity capable of imparting the same colour to Folin's reagent, the substance which had migrated in each experiment, either to the electro-positive or the electro-negative chamber, was tested pharmacologically by independent workers. Where 'adrenaline' was detected and estimated by the chemical method, its presence was confirmed in every instance by pharmacological tests. The tests were carried out by the usual standard pharmacological method, employing the decerebrated male cat, by Dr. K. Venkatachallam and Mr. A. N. Ratnagiriswaran of the Pharmacological Research Unit, Medical College, Madras. We very gratefully acknowledge their help.

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THE TRANSFORMATION OF CAROTENE INTO VITAMIN A IN LIVER AUTOLYSATES.

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THE demonstration of the transformation of carotene into vitamin A has up to the present not been conclusively made although there is no doubt that in the living organism such a change takes place. That the liver is the main organ concerned appears likely in view of the capacity of that organ to take up carotene given by mouth or injected into the circulation. *In vitro* experiments with liver hash or extracts incubated with carotene gave conflicting results.

Rea and Drummond (1932) failed to show the presence of vitamin A in the livers of cats and rats killed 72 hours after the injection of carotene into the portal vein. *In vitro* experiments with liver and carotene incubated over a period of time also failed to give positive results.

Ahmad (1931) working with rats also failed to obtain any transformation of carotene in liver-incubation experiments. Drummond and McWalter (1934) showed that carotene that had been injected into the portal circulation was taken up by the livers of rats and tended to disappear within a few days. The disappearance of the carotene could not, however, be correlated with the formation of vitamin A. Olcott and McCann (1931) on the other hand showed by spectrographic methods the appearance of vitamin A in an incubated mixture of rat-liver extract and carotene. No quantitative data however were given. Pariente and Ralli (1932) prepared a carotenase from dog's liver which, when incubated with carotene, produced a substance giving the antimony-trichloride test.

The inconclusiveness of the results obtained so far suggested to us a method which might avoid to a certain extent some unknown factors associated with

both *in vivo* and *in vitro* experiments. The method of sterile liver autolysis appeared to be a profitable line of investigation. In such an experiment the liver or a part of it is removed from the animal aseptically, plunged into paraffin wax (m.p. 38°C.), cooled quickly and left to autolyse at room temperature over a period of time. The advantage of such a method lies in that any vitamin A which may be formed or any carotene already present cannot be carried away by the circulation as in *in vivo* experiments. On the other hand the process of transformation may be dependent on the integrity of the liver cells, a feature which is absent in organ extract preparations but preserved to a certain extent in autolysis experiments. The animals employed in these experiments were the rabbit and rat. The former was preferred owing to its having a larger liver which could be cut into several portions and the time course of the transformation could be observed in one organ. The animals were fed a vitamin-A deficient diet until they lost weight or showed signs of vitamin-A deficiency. They were then given carotene by mouth or intravenously and killed 21 to 72 hours later. The liver was removed aseptically, cut into three or four pieces, each piece quickly dipped into alcohol to ensure sterility and plunged into the liquid paraffin wax. One portion was immediately weighed and its carotene and vitamin-A content estimated while the others were kept for varying periods at room temperature (about 78°F. to 85°F. during the monsoon in Calcutta). In only one case did putrefaction occur which was associated with a complete disappearance of both carotene and vitamin A. Similar autolysates of the spleen and kidneys were made with negative results. In the rat series, owing to the smallness of the liver, a portion of the organ from two animals had to be pooled for each analysis. The carotene and vitamin A were extracted by grinding the livers with anhydrous sodium sulphate and extracting with ether. This extract was then divided into two parts each of which was evaporated to dryness. One part was taken up in petroleum ether for estimation of carotene by the Lovibond tintometer; the other was taken up in chloroform for estimation of vitamin A by the antimony-trichloride reagent. The results are expressed in milligrams of carotene and blue units of vitamin A per gramme of liver tissue.

The results obtained by this technique are given in the Table, experiments I, II, III, IV, and V. In experiments I to III with rabbit livers an increase in blue units was associated with a decrease in the carotene content.

TABLE.

Experiment.	Number of days' autolysis.	Carotene mg. per g. of liver.	Blue units per g. of liver.
I.—Rabbit fed carrots and killed 3 days later.	0	0.0017	11.53
	10	0.0014	24.60
	17	0.0007	34.60
	28	Nil	37.20

TABLE—conclld.

Experiment.	Number of days' autolysis.	Carotene mg. per g. of liver.	Blue units per g. of liver.
II.—Rabbit fed 10 mg. carotene in oil. Killed 24 hours later.	0	0.0012	0.25
	3	0.0004	1.37
	10	0.00035	1.89
	17	0.00015	2.22
Putrefaction	28	Nil	Trace.
III.—Rabbit given 5.4 mg. carotene intravenously. Killed 24 hours later. 51.5 per cent recovered in liver	0	0.0860	14.66
	3	0.0527	20.21
	10	0.0367	26.94
	17	0.0272	36.69
	28	0.0182	55.66
IV.—Two rats—2.5 mg. carotene in oil fed to each. Killed 24 hours later.	0	0.00110	Trace.
	14	0.00045	Nil.
V.—Two rats—2.5 mg. carotene in oil fed to each. Killed 24 hours later.	0	0.00083	Nil.
	14	0.00060	Trace.

In experiment I which was of a preliminary nature no account was taken of the amount of carotene fed; it must however have been at least 30 mg. to 40 mg. The total amount in the liver at death was only 0.15 mg. During the 28 days' autolysis the concentration of carotene fell from 0.0017 mg. per gramme of liver to nil, while the blue unit rose from 11.58 to 37.2 per gramme of tissue. In experiment II the carotene was fed dissolved in oil and the animal killed 24 hours later. The concentration of carotene in the first sample of liver was not much less than that found in experiment I, while the blue units were almost nil. At the end of 17 days' autolysis the blue units increased to only 2.2 per gramme, while the carotene diminished to one-tenth its initial value. In this experiment less time had elapsed between the administration of carotene and killing the animal than in experiment I. This probably accounted for the low initial blue-unit value and it is possible that the carotene had not had sufficient time to become transformed into some precursor or form some intermediate compound preparatory to its transformation into vitamin A. In experiment III 5.4 mg. carotene were injected in aqueous suspension intravenously and the animal killed 24 hours later. In this experiment the total carotene recovered in the liver was

51.45 per cent, the initial concentration of carotene was relatively higher, 0.086 mg. per gramme and the blue units 14.6 per gramme of liver tissue. By the 28th day it will be seen that the carotene concentration per gramme of liver has fallen to 0.018 mg., while the blue units have risen to 55.66 per gramme of tissue. Experiments IV and V with rats were negative although a slight fall in carotene was observed. This is in keeping with the observation of Rea and Drummond (*loc. cit.*) and Ahmad (*loc. cit.*) who failed to obtain evidence of the transformation in rat livers. The former suggest, however, the advisability of repeating the experiments on the livers of herbivorous animals, a suggestion which has been justified by the experiments recorded here. It would appear that an enzyme or enzyme system is responsible for the transformation as the liver cells cease to live in the anærobic process of autolysis.

CONCLUSIONS.

1. The liver appears to be capable of transforming carotene into vitamin A during the process of aseptic autolysis.
2. An enzyme is probably responsible for this reaction.
3. There is no evidence that the spleen or kidney can effect such a transformation.

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STUDIES IN VITAMIN C.

THE EFFECT OF COOKING AND STORAGE ON THE VITAMIN-C CONTENTS OF FOODSTUFFS.

BY

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ALTHOUGH in recent years the vitamin-C contents of natural foodstuffs of India, both animal and vegetable, have been assayed by a number of investigators, very little work appears to have been done on the effect of cooking on the vitamin-C contents of these foodstuffs. Chakraborty (1936) has studied the effect of boiling on the vitamin-C content of milk and has found that boiling once reduces by half the vitamin-C content of milk. Pal and Guha (1937) have made a nutritional study of some cooked Bengalee dietaries without going into the question of the vitamin-C content of these dietaries. Chakraborty and Roy (1936) have made a study of the excretion of vitamin C in the urine of two healthy Bengalees on diets differing widely in their protein and fat contents in the form of meat and casein and butter, but made no assay of vitamin-C content of these cooked dietaries. This, in the opinion of the present author, is very essential in such a study, since the amount of ascorbic acid ingested daily varies widely with the form of the diet.

It has been shown by the author (Rudra, 1936) that the vitamin-C content of fruits and vegetables gradually diminishes on storage, the decrease in concentration starting at the skin. Ranganathan (1935, 1936) also has studied the effect of storage at room temperatures on the vitamin-C content of fruits and vegetables. Ghosh and Guha (1936) have studied the effect of storage at 0°C. on the vitamin-C content of some food materials.

OBJECT OF THE PRESENT INVESTIGATION.

In the present investigation the effect of cooking and storage under refrigeration on the vitamin-C content of both animal and vegetable foodstuffs has been studied. Such a study is important from the nutritional point of view as many vegetables are cooked before they are eaten, especially in India, and animal foods are eaten only after cooking. The use of electrical refrigerators in the home has grown and many foodstuffs are now stored under refrigeration before use.

INFLUENCE OF HISTAMINE AND ACETYL-CHOLINE ON INTESTINAL MOVEMENTS*.

BY

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IN view of the possibilities of the formation of histamine and acetyl-choline in the wall of the gastro-intestinal tract in small or large quantities and in view of the therapeutic value of acetyl-choline in cases of paralysis of the intestine, a thorough investigation of the nature of their actions on the bowel seems necessary. As the investigation so far carried out does not seem to be very exhaustive particularly in the case of acetyl-choline, it was considered worth while to examine the effects of these substances on the small intestine as a continuation of the work already carried out by the author (Krishnan, 1932-1934) on intestinal movements.

The influence of histamine and acetyl-choline on the movements of the small intestine was studied in about 25 cats and 2 dogs by either injecting the drugs intravenously into chloralosed animals and observing the effects on the movements of the intact bowel recorded by the balloon method or by adding the drugs to the bath of oxygenated Locke's fluid maintained at 38°C. in which the excised segments of small intestine were suspended and noting the effects on the movements recorded by Brodie's levers attached by thread to the upper ends of the segments.

Histamine.—It was Dale and Laidlaw (1910) who first observed that large doses of histamine given intravenously caused vomiting and purging in cats and that small strengths of 1 in 500,000 caused stimulation of an isolated segment of the jejunum which was not abolished by atropine. Olive Crona (1920) reported that large doses of histamine caused inhibition of the tonus and the rhythmical contraction of the small intestine in cats after an initial transient increase in tonus. Ivy and Vloedmann (1923) found no effect on the stomach and the intestine of dogs after small intravenous doses of histamine. Gasser (1926), Bishop and Kendall (1928), Gruber and Robinson (1929), all reported stimulating effects of histamine on the bowel. Mackey (1930) found the effect of histamine most marked

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in the ileum and noted that a strong initial contraction was followed by inhibition and later there was recovery sometimes with increased tonus. Atropine diminished but did not abolish the histamine contractions. Bernheim (1931) found in guinea-pigs that the decrease brought about by atropine in histamine contractions was inversely proportional to the ratio of histamine to atropine and concluded with Blockson (1932) that the action of histamine is both on the smooth muscle and on the parasympathetic nerve-endings. Tidmarsh (1932) reported that histamine in intravenous doses of $\frac{1}{4}$ mg. to $\frac{1}{2}$ mg. produced motor response in all parts of the large intestine of cat and that atropine in doses of 2 mg. reduced but did not abolish such responses.

In my experiments, histamine given intravenously into chloralosed cats in doses of 0.2 mg. to 0.5 mg. or added in doses of 1 mg. to 6 mg. to a bath of oxygenated Locke's fluid (100 c.c.) in which excised segments of small intestine were suspended, caused contractions in a motionless bowel or augmentation of movements already present in the bowel. In some cases of intact bowel there was a delay of 1 to 2 minutes after injection of histamine before the stimulating effect on the bowel could be noticed. These contractions were often followed by complete relaxation both in the intact and the isolated bowel for short or long periods and later there was recovery of tonus. These results more or less confirm the observations already made by Olive Crona and Mackey. Histamine was found to have an equal stimulating effect on all parts of the small intestine both in the intact and the isolated bowel. This finding does not corroborate Mackey's observation that the action of histamine is most marked in the ileum. With larger doses of histamine, contractions of the isolated bowel were stronger but slower. Atropine following histamine in equal or less quantity caused definite inhibition of the contractions, and after a short interval the contractions were resumed both in the intact and the isolated bowel. The effect of atropine was not a decrease in the strength of contractions as reported by Mackey, Bernheim, and Tidmarsh but a complete inhibition of the tonus and relaxation. The finding of Dale and Mackey that atropine does not abolish histamine contractions of excised segments is not borne out by my results. After the inhibition brought about by the first dose of atropine, subsequent larger doses of atropine had sometimes a stimulating effect. When histamine was administered after atropine the results were varied. When inhibition was present after atropine (1 mg.), histamine (0.2 mg.) caused contractions, while 0.5 mg. of histamine caused relaxation of the bowel which was contracting rhythmically after 3 mg. of atropine. Histamine also inhibited the stimulating action of barium chloride.

The above results corroborate the view of Bernheim and Blockson that histamine has its action not only on the smooth muscle of the intestine, but also on the parasympathetic nerve-endings in the wall of the intestine. This would mean, according to the humoral theory, that histamine is responsible for the liberation of acetyl-choline which will have a further stimulating effect on the intestinal muscle and the action of atropine added would be to neutralize the effect of acetyl-choline formed. The finding that large doses of atropine caused augmentation of the contractions is in support of the observation made by Magnus that in animal experiments large quantities of atropine tend to increase the peristalsis from some action exerted on the Auerbach's plexus and Cushny's statement that this increased peristalsis may possibly account for the vomiting.

similar increase in tonus and amplitude of contractions, but there was no initial depression observed in the intact bowel nor any periodical variation in tonus and rhythmical contractions. Addition of larger doses, 0.25 mg. or more, caused contracture of the segment of intestine. A previous dose of eserine increased the stimulating effect of acetyl-choline and a dose of atropine following acetyl-choline abolished the effect.

The fact that acetyl-choline acts as a stimulant to the intestinal muscle has been well established by the previous observers and is also borne out by the results of my experiments. But that there is an initial depression in the intact bowel and not in the isolated bowel, varying in duration according to the strength of the dose, has not been mentioned in the literature available so far. This depression is more pronounced after vagal section. In view of the recent findings of Feldberg and his co-workers (1934, 1935) that acetyl-choline is formed not only in the medulla of the suprarenal gland but also in all the sympathetic ganglia and is responsible for the adrenaline output and the stimulation of the post-ganglionic sympathetic fibres, this depression of the intestinal movements is easily explained as due to the sympathetic effects and adrenaline production caused by the dose of acetyl-choline injected. The depression is later overcome by the direct stimulating effect of acetyl-choline on the intestinal muscle. The more pronounced depression that resulted, when acetyl-choline was injected after the severance of the vagi, can be explained as due to the loss of vagal tone and the unopposed action of the sympathetic and the adrenaline produced.

In view of these observations, it is to be expected that, when acetyl-choline is used as a therapeutic agent, there will be an initial depression of the movements and some delay before the stimulating effect is obtained.

In carrying out the experiments on the intact bowel, the carotid blood pressure was also recorded. Smaller or larger doses of acetyl-choline caused a fall of blood pressure. The reversal effect on blood pressure after atropine was observed only when larger doses of acetyl-choline were given. After 1 mg. atropine 0.02 mg. of acetyl-choline was not effective but 0.2 mg. to 0.4 mg. of acetyl-choline caused a rise of blood pressure. These results are only in confirmation of the observations already made by Dale, Feldberg, and others. The rise in blood pressure is found to be very little or absent if the acetyl-choline is preceded by atropine and ergotoxin, showing thereby that, when the vaso-constrictor fibres of the sympathetic are put out of action by ergotoxin, the reversal effect of acetyl-choline on blood pressure after atropine is not seen.

SUMMARY.

1. The stimulating effects of histamine and acetyl-choline on the small intestine observed already by various workers are confirmed in cats and dogs.

2. It was found that histamine has its stimulating effect on all parts of the small intestine intact or isolated to the same extent and that atropine caused definite inhibition for short or long periods both in the intact and the isolated bowel. These results do not corroborate Mackey's observation that the action of histamine is most marked in the ileum and the findings of Dale and Mackey that atropine does not inhibit the stimulating action of histamine on excised segments.

3. Histamine is found to have its maximum stimulating effect when the tone of the muscle is low by its direct action on the muscle and by its stimulating effect on the parasympathetic nerve-endings causing thereby production of acetylcholine which is also a stimulant to smooth muscle.

4. As in the case of histamine, acetylcholine is found to have its stimulating effect on all parts of the bowel and not only on the lower part of the intestine in cats as observed by Bernheim.

5. There is an initial depression or inhibition of the movement of the intact bowel before the motor response appears after the injection of acetylcholine. This observation is not found in the literature available so far. The initial depression or inhibition is considered to be due to the adrenaline output and excitation in the sympathetic ganglia caused by the acetylcholine injected.

6. It is pointed out that, when acetylcholine is used as a therapeutic agent, some delay is to be expected before the stimulating effect is obtained.

7. The reversal effect on blood pressure after atropine was obtained with larger doses of acetylcholine as already observed by Dale, Feldberg, and others. The rise of blood pressure was very little or absent when both atropine and ergotoxin were injected previously.

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100 *Influence of Histamine and Acetyl-Choline on Intestine.*

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A PRELIMINARY NOTE ON THE PRESENCE OF A
HISTAMINE-LIKE BODY IN THE AQUEOUS
HUMOUR IN GLAUCOMA ASSOCIATED
WITH EPIDEMIC DROPSY.

BY

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EPIDEMIC dropsy is very common among the rice-eating population of Bengal. A number of outbreaks has occurred and each epidemic brings forth its own peculiarities; cases of one year greatly differ in severity, signs, and symptoms from those of another year. Among the varied features of the diseases, primary glaucoma, of late, has attracted much attention. Glaucoma associated with epidemic dropsy was first described by Maynard (1909) in a report of 100 cases which occurred during the outbreak in the year 1908-09. Mukherjee (1927) made observations on 253 cases in Bengal. During the outbreaks in 1929, 1930, 1932, and 1933, quite a large number of cases of glaucoma occurred; 325 of these patients from different parts of Bengal were treated by Lieut.-Colonel Kirwan in the Medical College Hospitals, Calcutta.

The glaucoma associated with epidemic dropsy is of the primary non-inflammatory type. Increased intra-ocular tension usually occurs as a late manifestation of the disease, but in the epidemic of 1932-33 quite a large number of cases of glaucoma was seen with slight or no other general signs of the disease.

The outstanding feature of this variety of glaucoma is the extremely high tension, rarely below 50 mm. of mercury. Tensions varying from 70 mm. to 100 mm. are quite common. In the Eye Infirmary of the Medical College, Calcutta, Kirwan (1936) treated a number of cases of glaucoma of this kind

by sclerocorneal trephining (Elliot's operation). The aqueous humour escaped under pressure and 0.25 c.c. on an average was collected and sent to this laboratory for biological testing. It is a curious fact that the toxin occurring in epidemic dropsy shows a special predilection for the intra-ocular capillary endothelium along with the general endothelial system of the body. Since the lesions manifested were of the same nature as those observed with histamine and allied substances, endeavour was made to demonstrate the presence of such bodies in the aqueous humour removed by operation.

EXPERIMENTAL.

The experiments were carried out in Dale's perfusion bath. Isolated strips of rat's uterus (virgin) were perfused in the usual way with oxygenated Fliesch's solution and the movements were recorded by means of optical as well as ordinary isotonic levers. The quantity of the fluid removed by operation being very small, it was difficult to demonstrate the presence of any active substance by ordinary methods. By means of the optical system the element of inertia could be greatly eliminated and it was possible to record the movements on a magnified scale. The fluid removed from the anterior chamber of glaucomatous eyes of epidemic dropsy when added to the bath showed relaxation of uterine musculature (see Plate VII, figs. 1 and 2). This phenomenon was observed in only seven cases out of 23. It may be observed here, however, that in a number of these cases, especially those examined in the beginning, the quantity of the fluid obtained was so small that no definite reactions could be obtained, especially as the optical system of recording had not yet been developed. In spite of this, definitely positive results were obtained as those depicted in Plate VII, fig. 3. The concentration of a histamine-like body in the aqueous humour in the two cases tested in Plate VII, figs. 1, 2, and 3, worked out to be in the neighbourhood of 1 in 1,000,000.

After the above findings, six control experiments were carried out with the aqueous humours from non-glaucomatous eyes by exactly the same technique; a negative result so far as the presence of a histamine-like body is concerned was obtained in every case. These cases were selected as controls in order to eliminate the possibility of histamine being present as a result of injury to tissue during operation. The results of experiments are tabulated below. It is regretted that owing to very great difficulty experienced in obtaining sufficient quantity of aqueous humour in non-glaucomatous cases the full number of 23 control cases could not be done.

	Positive result.	Negative result.	Total.
Glaucoma of epidemic dropsy ..	7	16	23
Normal cases	<i>Nil</i>	6	6

Ant Chamber Fluid.



FIG. 1.

Isolated uterus of white rat

FIG. 2.

Isolated uterus of white rat



Ant Chamber fluid
0.25 cc

Histamine
1:1,000,000



FIG. 3.

Perfusion of virgin uterus of white rat. Figs. 1 and 2 recorded by means of optical lever, Fig. 3 by ordinary isotonic lever. Note marked relaxation of the tone of the uterine musculature on the addition of anterior chamber fluid.

Aqueous humours of epidemic dropsy glaucoma cases were also analysed for their protein content. The number of cases studied was eight only, the results of which are given below. For comparison six normal cases were also studied.

	Albumen in g., per cent.	Globulin in g., per cent.	Total proteins in g., per cent.	Globulin to albumen ratio.
Average for aqueous humour of epidemic dropsy cases.	0.023	0.010	0.0310	0.450
Average for aqueous humour of normal cases.	0.0042	0.0101	0.0143	2.701

From the above, the albumen to globulin ratio in the aqueous humours of normal subjects is found to be 0.44, whereas it became 2.3 in cases of epidemic dropsy glaucoma. The total amount of globulin does not, however, materially change. The albumen, therefore, increases more than five times in aqueous humour in epidemic dropsy as compared with normal. Besides this, in a number of cases of glaucoma of epidemic dropsy we found by methods of biological testing some other substance present in the aqueous humour which is not ordinarily found in this fluid. That this substance is histamine or a histamine-like body may be imagined from the similarity of the pharmacological action. Such substances, whatever may be the source of origin, gain entrance into the body, circulate in the system in sufficient concentration as to cause vascular disturbances which upset, in some way, the normal function of the capillaries. The increased permeability which results leads to the escape of protein and fluid in the tissue space. This glaucoma of epidemic dropsy is a part of the generalized capillary disturbances such as those manifested in the skin and other organs (Chopra *et al.*, 1935). The increased pressure is due to the dilatation of the capillaries of the uveal tract, followed by an altered permeability of the endothelium as a result of damage by histamine or a histamine-like body. This leads to an increased output of fluid in the anterior chamber of the eye together with leakage of the substance which causes the damage. The altered permeability further helps the passage of protein bodies into the anterior chamber. The albumen should evidently pass out more freely because of the smaller dimension of the molecules. Once the albumen is there, it increases the osmotic pressure and helps to drain the fluid further into the anterior chamber. All these facts aggravate the symptoms of glaucoma in epidemic dropsy.

The pathologic process of the other varieties of primary glaucoma is probably different from that associated with epidemic dropsy.

SUMMARY.

Aqueous humour from 23 cases of glaucoma produced in epidemic-dropsy method was examined on the isolated rat's uterus in the Dale's perfusion bath.

In seven cases out of 23 definite histamine-like effects were obtained such as that represented in Plate VII. Fluid from six normal cases gave negative results.

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OBSERVATIONS ON THE BLOOD-LIPOID CHANGES IN OPIUM ADDICTS.

BY

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FROM a study of the changes in the physical properties of the blood sera of opium addicts Chopra *et al.* (1935) found that the pseudoglobulin fraction was considerably diminished while a rise in the euglobulin fraction occurred. From Chick's (1914) observations that euglobulin is an adsorption compound of pseudoglobulin with lecithin they thought it probable that the increased euglobulin formation necessitated a drainage of lecithin from the nerve cells, thus accounting for the degeneration of the nervous tissues observed in opium addicts. From a cytopathological study Ma (1932) found the Golgi apparatus in the tissue cells of the addicts to be altered from the normal in as much as, unlike the normal subjects' cells, these do not take the neutral red stain. The administration of lecithin which has been found by him to cure the withdrawal symptoms enables these cells to take this stain and to come back to their normal conditions. All these point to a disturbance of lecithin balance in the system.

As the two lipoids, lecithin and cholesterol, are more or less intimately associated in the system, especially in the blood, and as they sometimes may have antagonistic action, we took up this investigation with a view to determine the lipid contents of the blood of addicts when they are taking the usual doses of opium, during abstinence and again during and after the usual course of lecithin treatment when the withdrawal symptoms have disappeared.

The first difficulty we encountered was with respect to the method for the estimation of lecithin in the blood. We tried some of the well-known techniques but these failed to yield reliable results in our hands. After repeated trials we succeeded in evolving out a technique which gives very satisfactory results. The details of this procedure are discussed elsewhere (Chopra and Roy, 1936), but for the sake of convenience a bare outline is given here.

An accurately measured amount of the blood (say 1 c.c.) is extracted by means of alcohol-ether mixture according to Bloor's technique (1918). A measured amount of this filtered extract (10 c.c.) is then transferred to a 8" \times 1" Pyrex test-tube and evaporated to dryness in a boiling water-bath. One c.c. of a mixture of seven parts concentrated sulphuric acid and three parts of nitric acid are then added and the tube is immersed in a glycerine bath, which is set at around 180°C. and heated until no more nitrous fumes are observed. At this stage Merck's perhydrol is added slowly from a pipette, a drop at a time. Usually four to five drops suffice. Heating is continued until all water vapour is driven off and all traces of 'perhydrol' are removed. The mixture after digestion should be perfectly clear. It is then transferred completely to a test-tube 7" \times $\frac{3}{4}$ " graduated at 15 c.c., 20 c.c., and 25 c.c. by two lots of 5 c.c. distilled water. Into another similar test-tube, 5 c.c. of the standard phosphate solution containing 0.05 mg. of phosphorus and 5 c.c. of distilled water and 0.5 c.c. of concentrated sulphuric acid are placed; then to the standard and unknown, 1 c.c. of 5 per cent ammonium molybdate solution and 1 c.c. of the hydroquinone solution (0.5 per cent hydroquinone in 15 per cent sodium bisulphite) are added mixed and the tubes are placed in a boiling water-bath for ten minutes. The tubes are then removed, cooled, proper dilutions of the standard and the unknown are made and compared in a colorimeter.

Cholesterol was determined according to the method of Myers and Wardell (1918) but, instead of plaster of paris, we used silica powder for the absorption of blood as suggested by Foy and Kondi (1935).

In most of the cases we made a determination of the morphine contents of the urine of addicts at all the different stages mentioned before and for a few days after the discontinuation of the lecithin treatment. The figures are not strictly quantitative, but they serve to give us a rough idea of the amount of morphine excreted in the urine.

DISCUSSION OF THE RESULTS.

From the Table it would appear that the values for lipid phosphorus during addiction to opium vary from 9.3 mg. to 11.7 mg. per 100 c.c. blood, which is about the same for a number of normal individuals, the variations in the latter case being from 9.8 mg. to 11.9 mg. per 100 c.c. blood. In this connection we would like to point out that this range is somewhat lower than the values obtained with respect to Europeans and Americans (12 mg. to 14 mg. per 100 c.c. blood). Whether this is due to a difference in the techniques employed or to any racial or climatic factor, we are at present not in a position to hazard an opinion. The fact that the blood lecithin after years of addiction to opium is not appreciably affected does not appear to us to be unusual and this is what one would expect.

The system tries to maintain its essential blood constituents at a more or less constant level. Any excess of any of these is removed either by excretion or dealt with in some other way and any depletion is made up by drawing upon other sources. That there is a degeneration of nervous tissues in opium addicts has been observed by several workers. It is probable that the requirement of lecithin for the formation of increased euglobulin in the plasma is furnished by the nerve cells which in consequence become more or less depleted of lecithin. As a consequence we should expect some rise in the lecithin content of the blood but the figures that we have obtained do not show any appreciable increase. This may be due to the fact that the increase in the euglobulin is so small that it does not make any appreciable difference in the total blood-lecithin content, although the amount may be quite sufficient as to produce changes in the nerve cells. In the majority of cases, there is a rise in the lecithin content during the withdrawal period. It is probable that this apparent increase may be due to a certain amount of anhydræmia which is known to follow the withdrawal of opium as a result of perspiration, excessive salivation or diuresis which constitute part of the withdrawal symptoms, or as a result of purgatives administered to relieve the withdrawal symptoms. During and after lecithin treatment there is always an appreciable increase of blood lecithin which perhaps returns to the normal level after the injury to the nerve cells has been made good. It may also be observed that the daily dose of opium which the addict takes as also the duration of addiction has no marked effect upon the lecithin content of the blood.

The variations in the cholesterol content are more irregular at all the different stages mentioned above. In some cases during abstinence there is a marked fall in the cholesterol content, while in a few instances there is even a slight rise. Barbour, Hunter and Richey (1929) have shown that there was a diminution of water content of the liver, in case of addiction to morphine which is more or less restored on withdrawal. This alteration in the water content of the liver is, however, likely to bring about certain changes in its function, especially in the secretion of the bile which serves as a means of excretion of cholesterol. On withdrawal, the water content of the liver tries to regain its initial value and therefore the liver probably begins its bile secretion in an efficient manner, thereby assisting in the elimination of cholesterol to a greater degree. This may help to diminish the blood cholesterol content. Moreover on the commencement of the withdrawal the addict becomes suddenly run down. He loses his appetite and eats very little. His state of general health becomes suddenly so bad for the time being that in some cases there is every likelihood of collapse, if proper precautions are not taken. In conditions of cachexia or reduced vitality again hypocholesterolaemia is found to occur as shown by Bloor (1916), Denis (1917), and various other workers. Then again there is a certain amount of anhydræmia consequent upon withdrawal and this will tend to raise the cholesterol content. It is likely that the cholesterol content of the blood is determined by the preponderance of one or other of these factors. The irregular blood cholesterol contents may thus find an explanation from the point of view of the complex factors influencing its concentration. The cholesterol figures taken as a whole fall within the normal range for Indians as determined by Boyd and Roy (1929).

With regard to the excretion of morphine in the urine, it appears that morphine could be detected in the urine for four or five days after the taking of the last dose

but the amount of morphine excreted seems to bear no relation to the dosage or to the duration of addiction. Sometimes with a comparatively smaller dose, the amount excreted is very significant; on the other hand, when a much bigger daily dose is continued for a longer period, no appreciable morphine is found to be excreted even during the first few days. We intend to deal with this aspect of the problem more fully in a subsequent communication.

TABLE.

Date.	Name.	Daily dose in grains and duration of addiction.	Lipoid P in mg. per 100 c.c.	Cholesterol in mg. per 100 c.c.	Stage when examination made.	URINE REPORTS.	
						Date.	Morphine content, mg. per cent.
5-9-36	B. Singh	33, 3 years	10·7	109·0	D. A.
7-9-36			10·8	85·7	D. W.
10-9-36	G. Singh	140, 8 years	9·8	160·0	D. A.
19-9-36			11·9	120·0	A. T.
11-9-36	N. Singh	108, 9 years	11·3	192·0	D. A.
13-9-36			11·3	150·0	D. W.	14-9-36	0·3
						16-9-36	Nil.
16-9-36			12·5	150·0	A. T.	18-9-36	Nil.
15-9-36	M. Singh	45, 8 years	10·1	104·3	D. A.	15-9-36	2·5
						16-9-36	0·5
18-9-36			10·4	106·6	D. W.	18-9-36	0·7
21-9-36			11·3	100·0	D. T.	19-9-36	Nil.
25-9-36			13·4	100·0	A. T.	21-9-36	Nil.
21-9-36	R. Singh	45, 8 years	11·3	111·9	D. A.	21-9-36	1·0
23-9-36			12·5	109·0	D. T.	22-9-36	0·7
						24-9-36	0·5
						25-9-36	A trace.
						26-9-36	Nil.

TABLE—*contd.*

Date.	Name.	Daily dose in grains and duration of addiction.	Lipoid P in mg. per 100 c.c.	Cholesterol in mg. per 100 c.c.	Stage when examination made.	URINE REPORTS.	
						Date.	Morphine content, mg. per cent.
29-9-36	M. Ram	40, 5 years	11.7	120.0	D. A.	29-9-36	1.5
						30-9-36	0.4
1-10-36	.		11.9	138.7	D. W.	1-10-36	0.4
						2-10-36	0.3
5-10-36			11.9	165.5	D. T.	3-10-36	Nil.
8-10-36			12.1	133.0	A. T.	5-10-36	Nil.
						6-10-36	Nil.
29-9-36	A. Singh	60, 1 years	10.4	96.0	D. A.	30-9-36	2.5
						1-10-36	A trace.
1-10-36			11.6	96.0	D. W.	2-10-36 to 6-10-36	.. Nil.
5-10-36			12.8	116.0	D. T.
8-10-36			12.7	..	A. T.
9-10-36	A. Khan	120, 12 years	11.4	..	D. A.	10-10-36	A trace.
11-10-36			12.0	104.3	D. W.	12-10-36 to 17-10-36	Nil.
15-10-36			11.9	100.0	D. T.
17-10-36			11.9	111.6	A. T.
9-10-36	G. Sing	90, 5 years	11.0	141.0	D. A.	9-10-36	2.0
11-11-36			11.9	126.3	D. W.	10-10-36	0.3
15-10-36			12.5	160.0	D. T.	12-10-36	Nil.
17-10-36			11.9	138.7	A. T.

TABLE—*contd.*

Date.	Name.	Daily dose in grains and duration of addiction.	Lipoid P in mg. per 100 c.c.	Cholesterol in mg. per 100 c.c.	Stage when examination made.	URINE REPORTS.	
						Date.	Morphine content, mg. per cent.
5-11-36	B. Singh	95, 5 years	9.3	150.0	D. A.	6-11-36	A trace.
7-11-36			10.6	120.0	D. W.	7-11-36	A trace.
10-11-36			10.6	104.3	D. T.	9-11-36	A trace.
16-11-36			11.0	192.0	A. T.	10-11-36	Nil.
6-11-36	J. Patro	45, 20 years	10.0	141.1	D. A.	6-11-36	A trace.
7-11-36			10.6	107.1	D. W.	7-11-36	A trace.
10-11-36			11.1	133.3	D. T.	9-11-36	A trace.
16-11-36			12.5	104.3	A. T.	16-11-36	Nil.
18-11-36	J. Singh	96, 15 years	10.7	..	D. A.	18-11-36	A trace.
19-11-36			11.1	..	D. W.	19-11-36	Nil.
1-12-36			10.8	..	A. T.	20-11-36	Nil.
						to 26-11-36	
25-11-36	A. Singh	60, 9 years	9.6	..	D. A.	26-11-36	0.3
27-11-36			12.1	135.5	D. W.	27-11-36	0.5
1-12-36			10.3	150.0	D. T.	30-11-36	0.5
7-12-36			12.0	133.0	A. T.	1-12-36	A trace.
						2-12-36	Nil.
						7-12-36	Nil.
27-11-36	S. Singh	80, 12 years	9.3	92.0	D. A.	26-11-36 28-11-36	0.3 A trace.
30-11-36			9.5	100.0	D. W.	30-11-36 to 2-12-36	Nil.
4-12-36			9.6	100.0	D. T.

TABLE—*concl'd.*

Date.	Name.	Daily dose in grains and duration of addiction.	Lipoid P in mg. per 100 c.c.	Cholesterol in mg. per 100 c.c.	Stage when examination made.	URINE REPORTS.	
						Date.	Morphine content, mg. per cent.
30-11-36	R. Ram	80, 14 years	10.8	169.0	D. A.	1-12-36	0.7
2-12-36			11.4	140.0	D. W.	2-12-36	0.3
						3-12-36	0.1
4-12-36			12.5	148.0	D. T.	4-12-36	Nil.
						5-12-36	Nil.
9-12-36	M. Singh	90, 12 years	10.1	120.0	D. A.	10-12-36	1.0
10-12-36			10.8	129.7	D. W.	12-12-36	0.5
						14-12-36	0.5
17-12-36			11.3	109.0	D. T.	15-12-36	0.5
21-12-36			12.5	102.8	A. T.	17-12-36 to 23-12-36	Nil.

D. A. = During addiction.
D. W. = „ withdrawal.

D. T. = During treatment.
A. T. = After treatment.

SUMMARY AND CONCLUSIONS.

- (1) In the majority of cases the blood-lecithin contents of opium addicts have normal values.
- (2) There is an increase of the lecithin content during and after the course of lecithin treatment and in the majority of cases during abstinence as well.
- (3) The cholesterol figures are somewhat irregular and seem to bear no direct relation to the actual condition of the addict or to the lecithin content. On the whole the figures are within the normal range for Indians.
- (4) Morphine is usually detected in the urine for four or five days after the taking of the last dose. The amount of morphine excreted seems to bear no relation to the amount of daily dose and to the duration of addiction.

ACKNOWLEDGMENT.

We wish to acknowledge with thanks the valuable help rendered by Mr. Bolai Ch. Das, B.sc., throughout this investigation.

Levy and Boyer, 1928 ; Kanao, 1928 ; Koller, 1926 ; Piness, Miller and Alles, 1930 ; Hartung and Munch, 1929, 1931 ; Guggenheim and Loffler, 1915 ; Buck, 1932 ; etc.). The majority of these compounds have also been subjected to careful pharmacological examination with a view to the elucidation of the relationship which might exist between the chemical structure and the physiological behaviour of these compounds (Chen, Wu and Henriksen, 1929 ; Chen and Chen, 1933 ; Swanson, 1932 ; Curtis, 1929*a* ; Tainter, 1933*a* ; Gunn and Elphick, 1934 ; Barbour, 1916 ; Epstein, Gunn and Virden, 1932 ; Hjort, 1934*a* and *b* ; etc.).

The present communication concerns itself with a study of 13 amines (listed in Table I) which are either homologues of ephedrine or closely related to it in chemical composition. Some of the compounds have already been reported upon by previous investigators, though not from identical viewpoints. A few appear to be studied for the first time. These include six primary amines which may be considered as derivatives of β -phenyl-ethylamine ($C_6H_5.CH_2.CH_2.NH_2$), four secondary amines including ephedrine, two tertiary amines, and one quaternary ammonium halide. Practically all the compounds are synthesized at the suggestion of Prof. B. E. Read either in the Peiping Union Medical College or in the Yenching University, Peiping. The methods and details have been published elsewhere (Feng and Wilson, 1930 ; Feng, 1932 ; Wilson and Sun, 1934). Phenylaminopropanol (racemic) has been obtained from Messrs. Sharpe and Dohme, Baltimore. Natural l-ephedrine, isolated from Chinese ephedra, 'Ma Huang', has been used throughout.

METHODS.

A number of experimental methods have been employed in the study of the so-called 'sympathomimetic' amines. The study of the blood pressure is the conventional method and has been most widely employed ; though not ideal from all points of view, it affords one of the best indices of activity of substances belonging to the amine group. The response is fairly constant and is sensitive enough to allow a quantitative appraisal of even very closely related substances by direct measurement of the effect produced. Combined with the methods of atropinization, ergotization, and cocainization, this method can further be utilized for a critical analysis of the mechanism of action as well. The hyperglycemic activity of these amines has recently been utilized by Anderson and Chen (1934) in their quantitative evaluation. The effects on isolated smooth muscles have been largely employed as a qualitative test, mostly to supplement the information already obtained by the pressor method. In the rather extensive literature on ephedrine and allied compounds, very few references are available where a quantitative comparison based on the response of the smooth muscle-organs has been attempted. One of the most important effects of the ephedrine group of bodies being on the smooth muscles of the body, it appears desirable that more information with regard to the degree of action be obtained. This study has been undertaken primarily with the above object in view. The changes in physiological action incident upon the alteration in the chemical constitution have also been noted and the correlation between them, as can be deduced from the more significant results, will also be pointed out.

TABLE I.

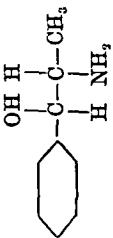
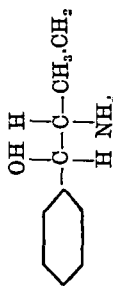
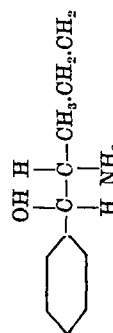
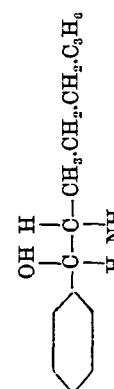
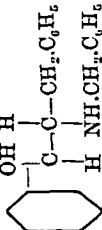
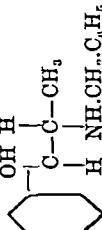
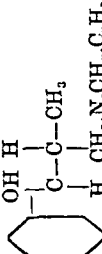
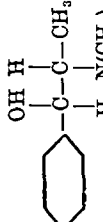
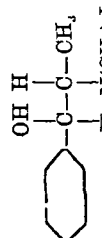
Number of compound.	Name of compound.	Empirical formula of hydrochlorides.	Structural formula.	M. P. of salt in centigrade.	Nature of rotation.
PRIMARY AMINES :					
1	Phenylpropanolamine (propadrin, nor-ephe- drin). Phenyl-1-amino- 2-propanol-1.	$C_9H_{11}NOCl$		178.0—179.0	Racemic.
2	Phenylbutanolamine. Phenyl-1-amino-2- butanol-1. (nor-homæphedrine).	$C_{10}H_{13}NOCl$		192.0—194.0	Racemic.
3	Phenylpentanolamine. Phenyl-1-amino-2- pentanol-1.	$C_{11}H_{15}NOCl$		..	Racemic.
4	Phenyl-octanolamine. Phenyl-1-amino-2- octanol-1.	$C_{14}H_{21}NOCl$		..	Racemic.

TABLE I—*concl'd.*

Number of compound.	Name of compound.	Empirical formula of hydrochlorides.	Structural formula.	M. P. of salt in centigrade.	Nature of rotation.
PRIMARY AMINES :					
5	Para-hydroxy-phenyl-1-amino-2-propanol-1. (Hydroxy-phenyl-propanolamine).	$C_6H_9NO_2Cl$..	Racemic.
6	Para-methylphenyl-1-amino-2-butanol-1. (Tolylbutanolamine).	$C_{11}H_{13}NOCl$..	Racemic.
SECONDARY AMINES :					
7	Phenyl-1-methylamino-2-propanol-1. (Ephedrin).	$C_{10}H_{15}NCl$		214.0—216.0	Natural levo.
8	2-Methylamino-1, 3-diphenyl-propanol-1.	$C_{18}H_{20}NOCl$		194.0—195.0	Racemic.

9	2-Benzylamino-1, 3-diphenyl-propanol-1.	$C_{22}H_{24}NOCl$		220.0—230.0	Racemic.
10	2-Benzylamino-1-phenyl-propanol-1.	$C_{16}H_{20}NOCl$		189.0—189.0	Racemic.
11	Benzyl ephedrine	$C_{17}H_{22}ON.HCl$		..	Racemic.
12	Methyl ephedrine. Phenyl-1-dimethyl-amino-2-propanol-1.	$C_{11}H_{18}NOCl$		188.0—189.0	Racemic.
13	Methyl ephedrine-methyl iodide.	$C_{12}H_{19}O.NI$		208.0—209.0 (uncorrected).	Racemic.

TERTIARY
AMINES :QUATERNARY
AMMONIUM
HALIDE :

Phenylpropanolamine (No. 1, Table I) has been chosen as the starting point of the primary amine group because it contains the 'phenyl-1-amino-2' arrangement of the side-chain which is present in ephedrine and epinephrine and which has been considered the optimum arrangement of the side-chain (Tainter, 1933b). This relationship is emphasized in the French literature where the compound is designated as 'nor-ephedrine'. Nos. 2, 3, and 4 belong to the same homologous series as No. 1, with gradually increasing number of carbon atoms linked to the terminal carbon atom. To study the effects of substituents in the nucleus itself, two members (Nos. 5 and 6),—a monohydroxy and a methyl substitution in paraposition—have been selected. Compounds with aryl and alkaryl groups have not received very much attention and this deserved further study (Nos. 8, 9, 10, and 11). Two tertiary and one quaternary ammonium halide have been added in the series to study the effects of transition from one group to the other. The number of compounds studied is small but it is felt that by choosing typical representative members of the various categories of amines, certain broad general principles might be deduced which may be helpful in future work.

The hydrochlorides of the bases have been used throughout. All the salts yield clear solutions in water at room temperature. Nos. 8 and 9 are comparatively less soluble than the others and in comparative experiments with equimolecular dosages quantitatively larger amounts of these solutions have to be used. Molar solutions and its fractions have been constantly employed. Concentrations have been calculated from the molecular weights of salts, and not of the bases.

TECHNIQUE.

Studies were made on the smooth muscles of isolated small intestines, uterii and bladder (urinary), and bronchioles of rabbits. In a small group of experiments, uterii of non-pregnant albino rats were also employed. Intestinal movements were recorded by Magnus' (1905) method. As different parts of the intestinal tract are known to respond in different ways to this group of drugs (Thienes, 1933), only segments from the first 25 to 30 inches of the gut (duodenum and jejunum) were used. In the large majority of experiments segments were removed, as needed, directly from the living urethanized animals (urethane 25 per cent 4 c.c. per kilo), the laparotomy wound being closed with clamps in the intervals. In other cases, a portion of the intestine was immediately removed from the killed animal, divided into small segments and transferred to long cylinders containing fully aerated tyrode solution at body temperature. Most of the experiments were done with non-pregnant uterii of rabbits weighing between 1.6 kilos and 2.2 kilos on an average. Small pieces of uterine horns varying from 2 cm. to 3 cm. were mounted following the details described by Broom and Clark (1924) and Patee and Nelson (1928) in their work on the standardization of ergot preparations by the 'rabbit uterus' method. The urinary bladders from the same animals were utilized, observations being made both on the fundus and the trigone. The effect on the bronchioles was studied in isolated lung preparations by the method of Sollmann and von Oettingen (1928) as modified by Pak and King (1930). Tyrode solution at room temperature was perfused and the drugs were injected directly into the inflow cannula. The drops were recorded both by means of a drop recorder and by direct counting.

The apparatus devised by Burn and Dale (1922), fitted with muscle-warming tubes (graduated) of 100 c.c. capacity, was used. Plain tyrode solution, freshly prepared* (pH 7.4 to pH 7.6), was employed in all the experiments. Oxygenation was effected by passing small bubbles of air (10 to 12 bubbles per second) through the tyrode solution in the reservoir as well as in the muscle troughs. By making a jet at the angle of the L-shaped glass bubbler, direct contact of the emerging bubbles with the immersed tissue was avoided. Change of solution was effected without exposing the tissue to outside air. The temperature was maintained fairly constant at $38^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$.

EXPERIMENTAL RESULTS.

1. *Isolated intestine. Rabbit.*—The results are summarized in Table II. Both qualitative and quantitative effects were observed. A very wide range of sensitiveness in response is presented by individual segments even from the same animal and under identical experimental conditions so that it is very difficult to lay down any standard concentration of the drug employed and the standard effects resulting therefrom. To obviate irregularities and to reduce inconsistencies in the results to a minimum, the plan adopted has been to carry out a large series of experiments, analyse the results mathematically, and to indicate the nature and degree of responses obtainable in at least 50 to 75 per cent of the experiments.

For quantitative evaluation, particular attention has been paid to the rapidity with which the inhibitory effect sets in, the extent to which normal rhythm is ultimately abolished, and the degree of recovery possible. The comparative strengths of individual members have been deduced from direct observations of the effects produced by equimolecular doses as well as the respective dosages required to produce the maximal response of a complete or nearly complete cessation of movements. The ratio of activity is expressed as the reciprocals of the doses required to produce equal submaximal responses as enunciated by Cushney (1908). As the nature of action of the various members of the group is markedly different, such evaluation of the respective activities is open to serious objection and is included as a rough approximation only.

A perusal of Table II shows that Nos. 1, 2, 3, 4, 5, 6, and 7 are predominantly inhibitory. This effect is elicited in the large majority of experiments when the

* Freshly prepared from stock solution as follows :—

Stock No. 1.			Stock No. 2.		
NaCl	1,000 g.	NaH_2PO_4	2 g.
KCl	25 g.	NaHCO_3	4 g.
CaCl_2	25 g.	Water to	8 litres.
(anhydrous).					
MgCl_2	12.5 g.	No. 1 keeps for a fairly long time. No. 2 requires more frequent changing.		
Water to	10 litres.			

SOLUTION :—

80 c.c. No. 1 water to	800 c.c.
Add No. 2	200 c.c.
	<hr/> 1,000 c.c. <hr/>

TABLE II.

Comparative effects of the amines on small intestines (rabbits).

Number of compound.	Minimum effective dose (100 c.c. bath).	Recovery rate.	EFFECTS ON			Comparative strength.	Ratio of activity (approx.), (Taking No. 1 as 1).	REMARKS.
			Tone.	Amplitude.	Rate.			
1	1 c.c. M/100 (1-55,000 approx.).	Spontaneous (30-40 secs.). Muscles responsive to barium.	Slight diminution. With large dose may be increased.	Very slight. With large dose definite decrease.	Not affected. Paralytic dose. Slight slowing at the end.	No. 2 > No. 1.	1	Action irregular. Predominantly inhibitory.
2	0.75 c.c. to 1 c.c. M/100.	Spontaneous (50-70 secs.) while drug still in bath. Responsive to barium.	Same as No. 1	Same as No. 1. Amplitudinal change slightly more marked.	Not affected except by paralytic dose.	No. 3 > No. 2.	1.2	Same as No. 1. Slightly more regular in action. Tone particularly affected.
3	0.3 c.c. to 0.5 c.c. M/100 (1-135,000 approx.).	Recovery helped after washing. Responsive to barium.	Initial fall. With large dose marked fall followed by a rise.	Fairly marked decrease.	Not affected except at the end.	No. 4 > No. 3.	3.75	Inhibitory effect more marked than No. 1 and No. 2. Recovery delayed.
4	0.25 c.c. M/100 (1: 160,000 approx.).	Recovery retarded (5-6 mins.). Responsive to barium in majority of experiments.	Diminution marked with large doses.	Diminished to almost nil.	Markedly slowed at the end.	No. 4 > No. 3 > No. 2 > No. 1.	20	Strong inhibitory action. Recovery delayed.
5	0.1 c.c. M/100 (1: 500,000 approx.).	Within 2 mins. after washing. Ineffective attempts at spontaneous recovery.	Marked decrease	Diminished to almost nil.	Slowed appreciably	No. 5 > No. 1 or No. 5 equivalent to No. 1.	10	Tonal effect prominent. Easy recovery after washing.
6	0.25 c.c. to 0.5 c.c. M/100 (1: 160,000 to 1: 80,000 approx.).	Within 1 min. after washing. Tendency to spontaneous recovery.	Fairly good decrease.	Diminished to nearly half.	Not affected. Slight slowing at end.	No. 6 > No. 2.	4.8	Same as No. 2. Only stronger effect.

7	0.5 c.c. to 1 c.c. M/100 (1-100,000) to 1-50,000).	Spontaneous while drug still in bath. Responsive to barium.	Very slight de- crease. Larger dose. More marked increase of tone.	Negligible diminu- tion.	Not affected	No. 7 stronger than No. 1 or equal.	..	Tonal effect promi- nent.
8	1 c.c. M/1,000 ..	Incomplete recovery; inhibition marked after first 4-5 mins	Not much affected	Initial increase. Slow and gradual diminution later.	Slowed slightly	..	12	Tone may be affected. Amplitudinal and rate changes prominent, gradual inhibition.
9	1 c.c. M/1,000 ..	Incomplete after washing. About 4-5 to revive.	Distinct diminu- tion of tone.	Distinct inhibition reduction to half or more.	Initially unaffected. Always slowed later.	..	60	Amplitudinal and rate changes more marked than tonal changes.
10	1 c.c. M/1,000 (1: 340,000).	Incomplete even after washing. 4-5 mins. or more to recover.	Not much affected until late.	Gradual and slow reduction much less marked than No. 9.	Always slowed later	Depression of a gradual nature. Muscle definitely affected.
11	0.5 c.c. to 1 c.c. M/100 (1-90,000 to 1-46,000).	Spontaneous (2-3 mins.) Responsive barium.	Diminished—may show transient initial rise.	Diminished—marked with large doses.	Slowed with large doses in 50 per cent experiments.	No. 11 stronger than No. 7.	..	Both tonal and amplitudinal chan- ges marked. Large doses—tendency to irregular contrac- tion.
12	0.5 c.c. M/1,000 (1-680,000).	Incomplete after washing. Muscle survives after 8-10 mins.	Initial increase followed by fall. Fall from the beginning.	Initial rise followed by marked reduc- tion. Large doses marked reduction leading to para- lysis.	Initially unaffected. Always slowed later. Complete cessation 1-3 mins.	No. 12 No. 10 No. 9.	300	Amplitudinal change more marked than tonal. Complete and long sustained relaxation.
13	0.5 c.c. to 1 c.c. M/100.	Tendency to sponta- neous recovery (2-3 mins.)	Decrease tone. Tendency to increase after 1 min.	Diminution very slight. Tendency to increase later.	Affected (slowed) in large doses.	Slight inhibition noticeable initially. Definitely muscular effect.

dosage level varies between the minimum and the optimum necessary to produce the typical effect. Nos. 3 and 4 often produce a transient primary stimulation with the minimum dose which is, however, rapidly masked by the inhibitory effect. A change from the purely inhibitory to purely stimulant action is often observed with Nos. 1, 2, and 7. This variability of the effect with varying doses appears to be the reason which has led many investigators not to recognize their essentially inhibitory effects but to take into consideration only their stimulant effects. Nos. 8, 9, 10, and 11 appear to be predominantly augmentor, noticeable only with very small doses. This is followed by marked inhibition which is long lasting and from which recovery of the intestine is much more difficult. Another interesting observation in this connection is that with Nos. 9, 10, and 11 the amplitude is much more affected than the tone, indicating probably a genuine muscular involvement. No. 12 differs from No. 7 in exhibiting a preliminary augmentor effect. No. 13 shows a predominantly augmentor effect though it is not uncommon to find in a few experiments a transient initial inhibition.

An attempt to study the mechanism of action of the various members, however, has not been very successful. That the inhibitory effects are not due to paralysis of the parasympathetics appears to be definite as no changes are observed before and after atropinization. With Nos. 10 and 11, however, atropinization sometimes abolishes the augmentor effect which points to parasympathetic involvement in these cases. The predominantly inhibitory effects seen with Nos. 1, 2, 3, 4, 5, 6, 7, and 12 indicate, from the almost immediate onset of the tonal response, a sympathetic stimulation, but no experimental evidence is available on this point. Ergotization is not of much help when the sympathetic endings are mainly inhibitor as is the case with the intestines. Apocodeine has not been used in view of the observation of Tainter (1933b) that the requisite degree of paralysis is not possible to obtain without serious involvement of the muscle. Responsiveness to barium has been employed to get an idea of the degree of paralysis of the musculature. The results obtained tend to indicate that Nos. 1, 2, 3, 4, 5, 6, 7, and 12 have all a mixed effect both on the sympathetic endings as well as on the musculature. The muscular effect appears to be more prominent with Nos. 3, 4, 6, and 12, whereas the nervous effect is more prominent in case of the others. With increase of dosage up to a certain limit, a transition from the purely inhibitory to a definitely stimulant effect may be seen with Nos. 1, 2, 5, and 7, where the muscular effect probably overcomes the nervous effect. The picture presented by Nos. 8, 9, 10, 11, and 13 is different from the rest in that the muscular stimulant effect is prominent here from the very outset. No. 8 diminishes both the tone and the amplitude, but Nos. 9, 10, and 11 have very little effect on the tone but marked effect on the amplitude and rate; all lead to a marked inhibition at the end only in different degrees. The muscular factor is obviously important though there is no evidence pointing to the absence of the nervous factor. The action of No. 13 simulates a weak nicotin effect on the intestines, the paralytic effect being completely absent. This is probably due to a weak stimulating effect on the sympathetic ganglia as has been shown by Pak and Read (1934).

2. *Isolated uterus. Rabbit.*—A critical comparative study was made on strips of non-pregnant rabbit uterus suspended in a Dale's uterine bath. Both qualitative and quantitative effects are noted. Two methods of comparison have

been adopted : (1) direct comparison on the same strip of uterus, keeping the doses constant and varying the orders of injection of the respective drugs ; (2) indirect comparison with adrenaline as a standard. Broom and Clark (*loc. cit.*) pointed out that the response of rabbit uterus to adrenaline is remarkably constant. This was confirmed by Patee and Nelson (*loc. cit.*). Following this principle, a constant response to a standard dose of adrenaline (after two to three injections) is obtained and the oxytocic response of the various members compared with this standard. The magnitude of the response has been determined by the accepted method of measuring the heights of the contractions of the uterine strips. This is quite satisfactory when the uterine strips are of the quiescent type but when they exhibit spontaneous or rhythmic activity, a fallacy creeps in because spontaneous contractions add greatly to the height of the ordinary response. In such cases, the 'sustained tonus increase' has also been taken into consideration as indicated by Thompson (1932). The presence of the well-known 'reversal' effect after ergotoxine has also been looked for. Ergotization was effected by adding ergotoxine ethanesulphonate in concentrations of 1 : 100,000 to 1 : 50,000.

The data available indicate clearly that there is a graded increase in oxytocic activity of the amines from Nos. 1 to 4. The relationship between Nos. 1 and 2 is not clearly defined in as much as they appear almost equivalent in potency in many instances. The stimulation following the injection of No. 4 is comparatively transient, leading on to a sharp and well-marked fall of tone from which recovery is not always complete. This points to toxic involvement of the muscle. Compared to Nos. 1 and 2, No. 3 appears to have more effect on the muscle as the recovery period is delayed, but the muscle is capable of regaining the original tone when the poison is washed away. The orders of activities of Nos. 1, 5, and 7, and Nos. 2 and 6 are again not conclusive. One would not be far wrong in judging No. 5 to be slightly stronger than both Nos. 1 and 7, while No. 2 is either slightly stronger or equal to No. 6. No. 12 appears to be definitely stronger than No. 7, a point which is of particular interest and will be referred to again. No. 13 is definitely weaker than No. 12. No. 11 is decidedly the most potent of the group consisting of Nos. 8, 9, 10, and 11 and all the members of this small entente are stronger than the other members of the series. Nos. 9 and 10 are probably equal with the scale slightly in favour of No. 10.

With regard to the nature of their action as elucidated by the ergotoxine method, not a single member can be classed in the same category as adrenaline, the typical sympathetic stimulator. No. 5 seems to be the nearest approach with No. 7 closely in its wake. Nos. 1, 2, and 8 show slightly diminished responses only. The rest of the series exhibit no change whatsoever.

3. *Urinary bladder. Rabbit.*—Young and Macht (1923) showed that the fundus of the urinary bladder is innervated by the parasympathetic and the trigone by the sympathetic. Macht (1926) later showed that various drugs react with these portions according to their affinity for one or the other type of N-endings and that the mechanism of action of an unknown drug can be elucidated satisfactorily in this relatively uncomplicated system. All the primary amines excepting No. 5 have a tendency to contract both the fundus and the trigone, the fundus effect depending largely on the functional state of the tissue. In a relaxed strip of fundus, there may not be any stimulation at all. The effect on the trigone appears to be more pronounced and regular with No. 2. No. 5

may bring about an inhibition of the relaxed fundus and a contraction followed by a gradual inhibition in a tonically contracted fundus. Ephedrine, as has already been pointed out by Macht (1929) and Liljestrand (1929), causes a contraction of both the fundus and the trigone. In small doses this effect appears to be more marked in the trigone, whereas in stronger concentrations, the reverse appears to be more true, indicating again a probability of a dual neuromuscular action of the drug. No. 12 behaves in the same way as No. 7 though the fundus effect is slightly more evident. Nos. 8 to 11 produce qualitatively the same response of an initial contraction of both the fundus and the trigone with a subsequent tendency to relaxation. No. 11 is again the strongest in potency, while No. 8 appears to be the weakest of this lot.

4. *Isolated uterus. Albino rat.*—The non-pregnant uterus of the albino rat is inhibited by adrenaline and is therefore a convenient tissue for differentiating between a 'sympathomimetic' action (Dale) and a direct stimulant action on the uterine muscle. From the results obtained, Nos. 5 and 7 appear to be definitely 'adrenaloid'. Nos. 1, 2, and 6 cause an adrenaloid response of a feeble character with small doses. With large doses, however, this effect may be completely reversed into a distinct stimulant effect. This is true more in the case of No. 6 than in Nos. 1 and 2. The rest are all definitely non-inhibitory. In the case of Nos. 9, 10, and 11, there may be inhibition as a late sequelæ following initial stimulation.

5. *Isolated lung perfusion. Rabbit.*—Ephedrine in a dilution of 1 : 1,000,000 to 1 : 200,000 causes an appreciable dilatation as evidenced by the increase in the number of drops. Propanolamine (No. 1) in concentrations of 1 : 200,000 to 1 : 50,000 has a tendency to constriction and so has No. 2. No. 11 in a dilution of 1 : 1,000,000 to 1 : 5,000,000 brings about an initial constriction followed, on increase of dosage to 1 : 100,000, by definite relaxation. Methyl ephedrine sometimes causes a slight constriction but generally a dilatation in dilution of 1 : 50,000. The variations in the number of drops from time to time with the injection of different amines are, however, so great that no quantitative estimation by this method appears to be promising.

DISCUSSION.

Certain parallel investigations with various members of the group of amines under study have been reported by a number of workers and it will be interesting and instructive to see how far the generalizations drawn from the present set of experiments agree or disagree with the conclusions arrived at by different methods of study. Our results have generally shown that the compounds in the series from 'propanolamine' to 'octanolamine' exhibit an increasing degree of activity on the rabbit uterus and perhaps also on the small intestine. From the effects on the bladder and the rat uterus, No. 1 appears to be more 'sympathicotropic' than the others, though No. 2 is a close follower. Nos. 3 and 4 appear to exert a toxic action on the musculature both of the intestines and the uteri but this toxic action is not so powerful as to cause a complete death of the tissues, as in many cases there is a revival after washing away the poison and the muscle is still responsive to barium. The order of activity of these four members bears out in full the impression of Chen, Wu and Henriksen (*loc. cit.*), that compounds with

long side-chains on the N-atom or on the α -carbon atom have comparatively greater oxytocic activity. It has, however, been shown that the optimum length of the side-chain is either two or three. Tainter (1933a) brings forth evidence on the basis of pressor response that lengthening the side-chain beyond 'propanol' decreases pressor response (as in butanolamine) and may further convert the pressor into a depressor response (as in No. 3, hexanolamine and No. 4). From our results on smooth muscles, it appears reasonable to conclude that lengthening the side-chain up to 'pentanol', at any rate, is not a marked disadvantage. Though a transition from 'propanol' to 'pentanol' is attended with a lessened sympathomimetic activity, the stimulant activity on the musculature is very definitely increased without any undue toxic action developing. The amines apparently produce varying degrees of response in different systems and the order of activity as manifested by their 'pressor' response need not hold good with regard to the smooth muscle effects. Differences are also noticed in their effects on blood sugar and toxicity tests on white mice. The blood-sugar response shows a graded increase (Anderson and Chen, *loc. cit.*; Tainter, 1933a) up to 'hexanolamine'. The toxicity of the compounds also rises up to 'pentanol' though 'octanol' is perhaps less toxic than all the rest.

The presence of an OH group in the paraposition in the nucleus appears to be an advantage in that the effect on the muscles of the intestines and perhaps uterus is slightly enhanced. This substitution certainly increases the sympathomimetic activity of the compound (results with rabbit bladder and rat uterus). The pressor action and the hyperglycemic action of this compound have also been reported to be stronger than its corresponding homologue. It may therefore be assumed that a monooxy substitution in the paraposition increases the activity of a compound. Such a general statement, however, is not always true, as there is decrease instead of an increase of pressor activity in a similar compound—tyramine ($\text{OH} \text{---} \text{C}_6\text{H}_4 \text{---} \text{CH}_2\text{CH}_2\text{NH}_2$) when compared to its homologue ($\text{C}_6\text{H}_5 \text{---} \text{CH}_2\text{CH}_2\text{NH}_2$).

The introduction of a methyl radicle in the paraposition of the aromatic nucleus does not seem to be attended with any consistent change in activity. Tolybutanolamine appears to be stronger on the intestinal musculature, but equal or slightly weaker in action on the uterine muscle than butanolamine. There is definite indication that the sympathomimetic action disappears to a great extent. This is in line with the findings of Alles (1933) who reports that methyl tyramine is equally effective as a bronchodilator and in some cases a more active bronchodilator. Substitution of a methyl group in the paraposition, on the other hand, is known to reduce the pressor activity to a fairly marked extent ($\text{CH}_3 \text{---} \text{C}_6\text{H}_4 \text{---} \text{CHOH.CH.CH}_3\text{NH}_2$ is $3/5$ as active as $\text{C}_6\text{H}_5 \text{---} \text{CHOH.CH.CH}_3\text{NH}_2$). With regard to the hyperglycemic responses the same relationship does not hold good. Anderson and Chen (*loc. cit.*) find that the addition of a methyl radicle in the paraposition increases the hyperglycemic activity of compounds. Hartung and Krantz (1931) also adduce evidence of increased hyperglycemia with methyl substitution of the aromatic nucleus.

The primary amines have a stronger pressor action than the corresponding methylated secondary amines (Hartung and Krantz, *loc. cit.*). Barger and Dale

(*loc. cit.*) also find that among sympathomimetic amines, a methylamino base is usually weaker than the corresponding amino base. In our experiments, however, there is very little indication that propanolamine is definitely stronger than ephedrine. Propanolamine appears stronger or perhaps equal to ephedrine in its uterine activity, whereas in intestinal activity ephedrine certainly is a more powerful inhibitory agent. This is not the only instance where a methylamino base behaves in such a way. A methylamino base may be actually stronger in activity or equal in activity to its corresponding amino base. Thus methylbenzylamine is more pressor than benzylamine and $C_6H_5.CH_2.CH_2.NH_2$ is equal in activity to $C_6H_5.CH_2.CH_2.NH(CH_3)$.

Secondary amines with substituted alkaryl or aryl or alyl groups at the N-atom or α -carbon atom from the N-atom (Nos. 8, 9, and 10) have been found, in our experiments, to be strong muscular stimulants, the ratio of activity being quite high in proportion to ephedrine. This stimulation, however, tends in a short time to lead to a well-marked relaxation, which is gradually more pronounced as one travels from the alkyl to the aryl substitution. These compounds also show a certain amount of toxic effect on the musculature of both intestine and the uterus as is seen from the incomplete and long-delayed recovery with even moderate doses. This supports the view of Chen, Wu and Henriksen (*loc. cit.*) that increase in the number of carbon atoms is not attended with corresponding advantage. The sympathomimetic activity also disappears perhaps completely.

Curtis (1929b) has shown that the conversion of secondary to tertiary amine of compounds belonging to the ephedrine series (by methyl or ethyl radicles) lessens their action on blood pressure. Effects on both the intestine and the uterus of methyl ephedrine have, in our series of experiments, given just the opposite results. Methyl ephedrine appears to be distinctly stronger in most experiments, while in others it is almost equal to ephedrine. The blood-sugar response indicates that the two members are almost equal. Methyl ephedrine has also been shown by Curtis (1929b) to be almost equal to ephedrine in its broncho-dilating power.

The other tertiary amine, benzyl ephedrine, has been found to be many times as active as ephedrine. Like all the other benzyl compounds, it is characterized by an initial stimulation followed by a lasting and prolonged relaxation. The sympathomimetic activity appears to disappear to a very large extent, if not completely, and, in certain experiments at least, there are indications of it stimulating the parasympathetics as well. The pressor response with benzyl ephedrine has been shown by Read (1932) to be predominantly depressor at the beginning followed by a pressor action above the original level.

Barger and Dale (*loc. cit.*) examined two quaternary ammonium salts, hordenine methiodide $OH \text{ } \langle \text{ } \rangle \text{ } CH_2.CH_2.N(CH_3)_3^+I^-$ and trimethylaminoethyl catechol chloride $(OH)_2.C_6H_3.CH_2.CH_2.N(CH_3)_3^+Cl^-$ and found both to possess a typical nicotin-like action. Our observations with methyl ephedrine methiodide fully bears out this observation. It has, however, a very weak nicotine action. It is certainly weaker than methyl ephedrine, the parent amine. Anderson and Chen (*loc. cit.*) have reported on a quaternary ammonium iodide and found it to be stronger than its corresponding homologue both in pressor action and in hyperglycemic activity. Hordenine methiodide has also been found to be about 50 times more active than hordenine itself in broncho-dilator activity.

From what is said above, the futility of making sweeping generalizations with regard to chemical structure and pharmacological action becomes at once apparent. While the order of potency for a given series of compounds may be quite constant for any one kind of pharmacological test, the sequence or degree of activity need not be and usually is not the same for other tissues of the body. The response of the amines at least seem to be entirely different with regard to pressor activity, hyperglycemic activity, smooth muscle effects, toxicity, etc. Further observation may reveal still other differences in their behaviour to the different tissues of the body.

SUMMARY AND CONCLUSIONS.

1. A group of 13 amines related to ephedrine have been investigated with reference to their effects on the smooth muscles of the small intestines, uterus, bladder (fundus and trigone), and bronchioles. Both qualitative and quantitative effects have been observed and a comparative estimate of the nature and degree of action of individual members attempted.

2. The compounds studied include six primary amines, four secondary amines, two tertiary amines, and one quaternary ammonium halide.

3. As the length of the side-chain is increased from propanolamine to octanolamine, there is a tendency to increase of activity on the plain muscles. Octanolamine, however, exerts a possible toxic action on the musculature. The sympathomimetic activity gradually disappears from Nos. 1 to 4.

4. The substitution of a monohydroxy radicle in the paraposition increases the strength of the compound and makes it more sympathomimetic.

5. The introduction of a methyl radicle in the paraposition is not of particular advantage. The intestinal effect may be more pronounced in certain cases. Such a substitution, however, removes the sympathomimetic activity of the compound to a large extent.

6. The conversion of a primary to a secondary amine may or may not diminish the activity of a secondary amine. Ephedrine may be actually more inhibitory on intestinal musculature than propanolamine, a primary amine.

7. The introduction of an aryl or alkaryl radicle in the N-atom or the terminal carbon atom increases temporarily the muscular activity of the compounds very greatly. This stimulant effect wears off after a short time leading to inhibition. The benzyl radicle brings about the relaxation at a much quicker rate than a phenyl radicle, which is characterized by a slow and gradual action. The sympathomimetic activity disappears almost completely with alkaryl or aryl substitutions.

8. The conversion of a secondary to a tertiary amine increases the activity on the smooth muscles. With a methyl substitution, the activity increases but slightly and the sympathomimetic efficacy becomes correspondingly slightly less marked. With a benzyl substitution, the activity increases markedly at the beginning followed by marked diminution. The sympathomimetic activity in aryl substitution perhaps disappears completely.

9. The quaternary ammonium iodide of methyl ephedrine is weaker than the parent amine. It has a nicotin-like action and does not seem to possess any sympathomimetic activity.

10. The amines apparently differ a good deal in their responses to different tissues. Very little similarity in the orders of activity amongst individual members is seen when their responses to blood pressure, smooth muscle effects, hyperglycemic activity, etc., are considered. Generalizations with regard to the effect produced by various substitutions in the ring and the side-chain of the benzenenucleus are therefore of limited value.

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CHEMICAL AND PHARMACOLOGICAL EXAMINATION OF *RANDIA DUMETORUM*, N. O. *RUBIACEÆ*.

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Mainphal or emetic nut is obtained from *Randia dumetorum* (Lam), a shrub cosmopolitan in the tropics and common throughout India. The dried fruit has been known to Ayurvedic and Unani pharmacists for a long time as an emetic and ecbolic (Dymock, 1891). It is described by the old Sanskrit writers under the name of *Madna* as pungent and dry, and beneficial in leprosy and phlegmatic swellings, the best and the safest of emetics. Mohammedan physicians of India have adopted it as a substitute for the *Jouz-el-kai* of Arabs and describe it as an emetic, at the same time acting as an aperient. Moodeen Sheriff (1869) describes the dry mucous pulp only as a good substitute for ipecacuanha in dysentery. Chevers, on the authority of Edgeworth, states that the fruit is used in Jullundhar as an ingredient in medicines given by the mouth for the purpose of procuring abortion (Waddell, 1928). Dymock and others have also mentioned it being used as a fish poison and to preserve grain from the attack of insects. Chopra (1933) has included it in the list of indigenous drugs worth investigation.

The earliest investigations date from the last quarter of the last century, and were confined to the chemical composition of the drug. Dymock and others found the fruit possessing, besides resin and colouring matter, an active principle, saponin, hydrolysed by acids into sapogenin and glucose.

From the preceding review of the literature, it will be observed that the statements respecting the physiologically active constituents of the fruit are rather vague. The work reported below was therefore undertaken.

Chemical composition.

The material employed for this investigation consisted of the brown kernel of the ripe sun-dried fruits obtained from a local Unani druggist.

A preliminary examination by the usual methods showed the presence of a reducing sugar: alkaloids, starch, and tannin were absent.

1. Steam distillation yielded a trace of a pale yellow oil having the characteristic odour of the drug.
2. Extraction with solvents: 25 g. of the ground material were extracted with various solvents successively in a Soxhlet apparatus when the following amounts of extracts, dried at 100°C. were obtained:—

	Per cent.
Petroleum ether (40°C. to 60°C.)	0.5
Ethyl ether	0.05
Chloroform	0.8
Ethyl acetate	1.0
Ethyl alcohol (strength 91 per cent) .. .	40.1
TOTAL ..	42.4

For a detailed examination, 1 kg. of the powdered pulp equivalent to 3 kg. of the whole fruit was thoroughly extracted in a continuous extraction apparatus with hot 90 per cent alcohol for about eight hours, which, on leaving overnight, deposited a voluminous amorphous precipitate. This was filtered off and the filtrate distilled under reduced pressure. The total brown viscous extract was then steam distilled.

Volatile constituents of the extract.

Essential oil.—The distillate was thoroughly extracted with ether and the ethereal extract, after extraction with dilute NaOH, left as residue about 5 g. of a thick greenish yellow oil having the characteristic smell of the drug.

Volatile fatty acid.—A portion of the distillate, on careful evaporation, left a little residue, acid in reaction, showing it to be a fatty acid. The quantity was too small for more detailed work.

Non-volatile constituents of the extract.—The dark-brown aqueous residue from steam distillation was slowly evaporated and the viscous mass was then taken up with dilute alcohol (90 per cent) leaving very little undissolved.

Saponin.—The alcoholic solution, on cooling, deposited a heavy amorphous precipitate which was filtered off (filtrate A).

The precipitate responded clearly to all the tests for saponins.

Purification of the saponins (Rosenthaler, 1930).—The crude residue was washed several times with ether, and dissolved in hot 75 per cent alcohol. The flocculent precipitate thus formed was removed by filtration of the hot solution through suction. The filtrate was poured drop by drop into a large volume of ether resulting in a brownish white flocculent precipitate, sticking to the sides of the beaker. This was filtered off and dried *in vacuo*. Its melting point was between 200°C. and 210°C.

Separation of the acid and neutral saponins (Hass and Hill, 1928).—*Neutral saponin.*—The residue was taken up with hot water in which it completely dissolved. The aqueous solution was treated with just sufficient neutral lead acetate solution to precipitate the saponins, briskly stirred, a little warmed, and filtered. The gelatinous precipitate, which was too difficult to be filtered, was centrifuged, the residue washed several times with water, and the washings and the filtrate collected (filtrate B). The dried gelatinous mass was then suspended in water and treated with dilute H_2SO_4 and filtered. The filtrate was freed from lead by passing H_2S and evaporated to the consistency of a syrup. This was boiled with 90 per cent alcohol. From this alcoholic solution the neutral saponin was precipitated by pouring it drop by drop into a large volume of ether and dried over H_2SO_4 . The resulting precipitate was further decolorized with animal charcoal; its melting point was between 230°C. and 240°C. (No sharp melting point could be determined.) The yield was about 30 per cent.

Acid saponin.—Filtrate B was then treated with basic lead acetate and the precipitate delead and the saponin purified as above. About 2 g. of brownish white material were obtained; its melting point was between 195°C. and 200°C.

Final purification of the neutral saponin.—To free the saponin from carbohydrates, colouring matter, and other colloids the *dialysis*, the *magnesia*, and the *salting out* by $(\text{NH}_4)_2\text{SO}_4$ methods were all tried and it was found that, at least in this case, the *precipitation method with ether* is the best and safest.

Acid resin.—Filtrate A was evaporated and the residue treated repeatedly with distilled water and filtered. The undissolved mass was finally taken up with dilute NaOH. The yellow alkaline solution was neutralized with dilute HCl, when a greenish white mass floated on the surface; this was collected and dried over H_2SO_4 . By its solubility in ether, alcohol, and NaOH, and its insolubility in water, it was confirmed as a resin.

Physical and chemical properties of the neutral saponin.—The so far purified neutral saponin is a white, brittle amorphous mass (melting point between 230°C . and 240°C .) soluble in water and cold dilute alcohol, and insoluble in benzol, petroleum ether, ethyl ether, acetone, and chloroform. The aqueous solution assumes a yellow tint and froths heavily on shaking and imparts a slightly alkaline reaction to litmus with a pH ranging between 7.0 and 7.8. The aqueous solution, 1 per cent, showed a specific rotation of -22° at 29°C .

The following colour reactions were obtained :—

1. Concentrated H_2SO_4 developed a red coloration in the cold.
2. Concentrated H_2SO_4 with a little FeCl_3 gave a bluish green colour.
3. Potassium ferricyanide containing a little ferric chloride gave Turnbull's blue reaction.
4. Ammoniacal silver nitrate was reduced to metallic silver.
5. Concentrated H_2SO_4 and phosphomolybdic acid did not produce any coloration but the saponin was hydrolysed.
6. Concentrated H_2SO_4 and tungstic acid—a deep red colour which became black afterwards.
7. Concentrated H_2SO_4 and vanadic acid—at first greenish yellow, finally blackish red.
8. Concentrated H_2SO_4 and phenol—pale yellow in the cold, changing to cherry red on warming.
9. Neutral lead acetate gave a voluminous precipitate of lead salt. Basic lead acetate did not produce any definite precipitate, but turbidity was noted after a short time.

Hydrolysis of the saponin—glucose and sapogenin.—On reflexing with dilute acids (10 per cent HCl or H_2SO_4), the saponin was hydrolysed and a white amorphous, flocculent insoluble precipitate was obtained which was filtered and washed several times with water and finally dried over H_2SO_4 —melting point between 200°C . and 210°C . (A sapogenin.)

The filtrate, on concentration, was subjected to Fehling test which was quite positive. An osazone was then prepared; its melting point was found to be between 200°C . and 202°C ., and it was completely soluble in acetone (glucosazone 203°C . to 204°C .). Saliva, emulsin, and prolonged boiling with H_2O also hydrolysed the saponin.

Ash.—6.3 g. of the dried (at 100°C .) seeds from the kernel were burnt to white ash (0.45 g.). The ash on qualitative analysis was found to be K_2O , Na_2O . Lead in very small quantity was indicated by a positive chromate test.

The shell and seeds were found to be free from saponin. [Moodeen Sheriff (*loc. cit.*) remarks that only the pulp possesses emetic properties.]

PHYSIOLOGICAL PROPERTIES.

- (1) *Taste.*—It has a slightly bitter taste and causes salivation.
- (2) *Irritation of the mucous membrane.*—When inhaled, it causes sneezing. A drop of 1 per cent solution causes redness in the rabbit's cornea.
- (3) *Hæmolytic index.*—(a) With whole blood (Hass and Hill, *loc. cit.*) a 1 per cent solution defibrinated sheep's blood in 0.9 per cent NaCl was used for this purpose.
 1/20,000 caused hæmolysis in 7 minutes.
 1/40,000 " " " 35 "
 1/80,000 did not cause hæmolysis even on keeping overnight.

(b) *Washed blood corpuscles.*—With 0·5 per cent suspension of washed blood corpuscles, the time of hæmolysis was shortened but the concentration remained practically the same.

1/20,000 hæmolysed in 5 minutes.

1/40,000 " " 30 "

1/80,000 did not hæmolyse even on keeping overnight.

The hæmolytic index is therefore 1/40,000.

(4) *Toxicity.*—

On paramæcia.

1/1,000 kills almost all the organisms.

1/5,000 kills only the larger individuals.

Fresh-water fish were killed by a concentration of 1/20,000 in about 12 minutes. 1/40,000 was not fatal.

(5) *Mammals.*—0·120 g. in 60 c.c. of water given by the stomach tube to a dog of 6 kg. body-weight brought about vomiting twice within six minutes, after which the animal soon became normal. 0·300 g. injected into the tibial vein in another dog of 5 kg. body-weight did not cause vomiting. This shows that the emetic and expectorant action depends upon local irritation of the stomach. 0·060 g. injected into the ear vein of a rabbit of 2 kg. body-weight in divided doses in the space of one hour did not prove fatal, but 0·095 g. brought about immediate death. In a blood-pressure experiment on a rabbit 0·190 g. was given in divided doses before death occurred: the blood pressure fell down about 30 mm. with each injection, but recovered in a couple of minutes. The fall of blood pressure was unaffected by administration of enough atropine to paralyse the vagal terminations. The same kind of a passing fall of blood pressure was seen in a dog. Death was brought about by cardiac arrest with the last large dose (0·300 g.) injected, a few gasping respirations occurring after the stoppage of the heart and fall of blood pressure to zero. In the rabbit, however, at the termination of the experiment, the bladder was full of red-coloured urine which showed the absorption bands of hæmoglobin; there was sanguineous fluid in the peritoneum and the lungs showed hæmorrhages under the pleuræ. In the case of the dog, the urine was clear.

(6) The pendulum movements of excised intestine of rabbit were rendered feeble and the tone lowered by a concentration of 1/50,000.

(7) The perfused heart of the frog was unaffected by a concentration of 1/100,000 but was arrested in a few minutes by 1/75,000, and practically instantaneously by 1/50,000, the ventricle going into systole, the auricles distended, while the sinus continued to beat feebly.

The detoxicating action of the liver was shown very strikingly, on perfusing through the anterior abdominal vein in a closed circuit arrangement holding 30 c.c. of fluid. With a solution of 1/50,000 and subsequently of 1/20,000 the heart was unaffected. The perfused solution which had passed through the liver on then being introduced directly into the inferior vena cava was seen to be devoid of poisonous action, while a fresh solution of the same strength (1/20,000) then brought about immediate arrest of the heart.

The material therefore appears to be one of the weaker members of the large group of saponins.

SUMMARY.

The alcoholic extract of *Randia dumetorum* fruits has been found to contain, besides the unidentified water-soluble fatty acids, an essential oil, green colouring matter, a neutral saponin, an acid saponin, and an acid resin.

The pharmacologically active constituent is a neutral saponin, behaving as one of the weaker members of the large group of saponins.

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PHYSICAL CHANGES IN BLOOD *IN VIVO* AFTER
INJECTION OF VENOM FROM INDIAN COBRA
(*NAIA NAIA VEL TRIPUDIANS*)
INTO MONKEYS.

BY

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THE hæmolytic action of snake venoms on red blood cells constitutes but one of its most important effects, the mechanism of which has unfortunately been still an unsolved problem. The action of venom on the blood has been observed to vary with the nature of the venom and also with the species of the animals on whose blood it acts. The conditions favourable for hæmolysis have also been found to differ in a similar way with the nature of the venom and the organism on which it acts (Martin, 1895; Kellaway, 1929; Kyes and Sachs, 1903; Lamb, 1905; Noc, 1904).

Kellaway and his co-workers have concluded that snake-venom hæmolysis is of the type of 'adsorption hæmolysis' according to the classification of Herman and Rohner (1925) resulting from the lysis of the lipoid or the protein-lipoid envelope of the red cells resembling lecithin more than saponin in its effects. They explained the serum inhibition of the venom hæmolysis on this basis and attributed it to the effect of changes brought about in the plasma as well as in the colloidal system composed of protein, lecithin, and cholesterol constituting the limiting surface of the corpuscle. But most of the work in this connection, as well as those of many others, have been done *in vitro*. Experiments *in vitro* in such cases, no doubt, make matters much simpler by eliminating many of the complex variables

that might otherwise come in but such simpler conditions certainly do not represent the actual state in a living body. Kellaway and Williams (1933) seemed to have understood this as they have made the introductory remark in one of their communications that if snake venoms were not so complex, the ideal method of study of the hæmolytic action would be by observation of the results produced by injection in living animals.

We thought it expedient to study the direct effect of the venom *in vivo* when injected into the anthropoids bearing fully in mind the complexities associated with such a study. The present paper embodies the results of our studies on the changes in some of the physical properties of the plasma and also of the surface condition of the corpuscle with a view to get a clue to an explanation of the hæmolytic property of snake venom, if possible.

EXPERIMENTAL PROCEDURE.

Cobra venom was dissolved in sterile physiological saline in strength of 1 in 10,000, i.e., 0.1 mg. per c.c., and was injected intragluteally into monkeys (*Silenus rhesus*). In the first monkey the dose injected was very small and in the subsequent ones it was gradually increased till we reached 0.01 mg. and 0.1 mg. per kilo body-weight, i.e., one to ten mouse units. These are the doses usually administered to man for therapeutic purposes. To each monkey two doses were given, the first being a very small dose, and the second a three times bigger dose, 24 hours later. In all the cases the blood was drawn from the saphenous vein by a 10 c.c. syringe, sterilized by boiling olive oil, immediately before giving the venom injection (as a control for normal blood) and two hours after the injection. A third specimen was drawn 24 hours later. Then a second higher dose was given and as usual 10 c.c. of the blood were drawn again after two hours and 24 hours, to study the effect of the venom immediately after its absorption and later on when it was being excreted.

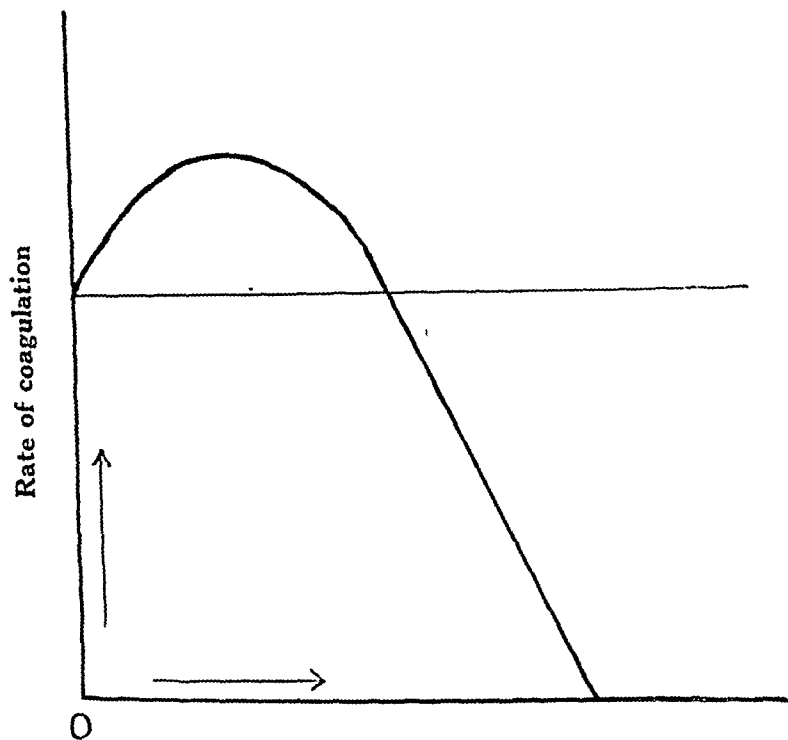
The blood drawn from each monkey was treated with a 2 per cent solution of potassium oxalate in the proportion of blood to oxalate as 10 to 1 by volume. A few drops of this oxalated blood were then mixed with an isotonic glucose solution buffered with phosphates. The migration velocity of red blood cells so suspended was then determined per unit potential gradient with the help of a micro-cathaphoretic cell. The remainder of the oxalated blood was centrifuged and the plasma carefully separated out. Physical properties, such as the surface tension, the relative viscosity, pH, and the buffer action of this plasma, were then determined by methods mentioned in our previous communications (Chopra and Choudhury, 1928).

It may be incidentally mentioned here that in this series of experiments we preferred blood plasma to blood serum for two reasons: firstly, it was observed that although blood from monkeys drawn before injection of venom would coagulate and express the clear serum the blood drawn after injection of venom, especially in larger doses, did not clot at all but formed a thick fluid mass.

With higher doses of venom, hæmolysis was found to take place, while at very low doses coagulation would occur as usual but it was only with moderate doses that a peculiar mass was obtained wherein the red cells did not actually hæmolyse as seen under the microscope but still refused to form the clot. We did not pursue our observations much further in this direction, but beginning from

very small concentrations of venom it was incidentally observed *in vitro* that the rate of coagulation was accelerated with increasing concentration of venom in the blood, and this persisted up to an optimal concentration where the rate was at its maximum. With further increase of venom concentration, the rate of coagulation was observed to slow down till at last, at a certain region depending upon both the specimen of venom and of blood, there was no clotting at all. At this stage a viscous fluid mass was formed wherein the red cells remained intact. This is illustrated in the Text-figure. With still higher doses the red cells haemolysed.

It may not be out of place to mention here that this peculiar behaviour of venom can be understood in the light of the considerations set forth by the senior author and his co-workers in some previous papers. It is quite likely that this behaviour on the part of the venom might be due at lower concentrations to the activity of the fibrin ferment that is present therein, whereas at higher concentrations the fibrinolytic action of the venom probably gains ground and destroys the fibrin that is responsible



Concentration of venom

TEXT-FIGURE.

for clotting (Chopra, Das and Mukherjee, 1936). Another factor that possibly acts along with this has been pointed out by Chopra, Chowhan and De (1935). This consists in the formation of some fine coagulum by the fibrin still present in the blood. (In spite of the depletion of fibrin by the proteolytic ferment present in the venom it is quite likely that there may always be a small amount of this protein in the blood as a result of two opposing influences that the venom exerts.) There is some evidence to believe that the coagulum thus formed sticks to the lumen of the blood vessels. Hence the blood that is drawn out is poor in fibrin and remains fluid. A drop of such blood examined under the microscope showed that the corpuscles were all intact and did not haemolyse.

The second reason for our preference of the plasma instead of serum consists in certain experimental conveniences. The preparation of the red-cell suspension for measurement of the migration of erythrocytes in an electric field necessitated

oxalated blood whereas for the expression of serum, blood without the anticoagulant is required. By selecting the blood plasma for our experiments we could have both red cells and plasma in one operation. The results have been given in the Table below :—

TABLE.

Action of cobra venom on the blood of monkeys in vivo, when injected intragluteally.

Monkey number.	Dose per kilo in mg.	Time.	Surface tension.	Relative viscosity.	pH.	Buffer action.	Mobility $\times 10^5$.
M. 1	0.000000	..	51.0	1.70	7.33	6.50	32.2
	0.000025	2 hours after	49.5	1.83	7.35	6.52	29.8
	"	24 " "	50.0	1.70	7.35	6.44	28.6
	0.000075	2 " "	49.6	1.71	7.31	6.48	27.0
	"	5 days "	50.0	1.64	7.30	6.57	25.3
M. 2	Normal	58.0	1.62	7.40	6.46	34.6
	0.0003	2 hours after	56.2	1.70	7.44	6.42	34.8
	"	24 " "	56.1	1.65	7.34	6.44	33.6
	0.001	2 " "	54.6	1.60	7.30	6.30	33.3
	"	24 " "	53.1	1.70	7.36	6.43	31.3
	"	72 " "	51.8	1.60	7.43	5.87	28.7
M. 3	Normal	59.4	1.54	7.31	6.49	28.0
	0.001	2 hours after	55.0	1.70	7.09	6.20	26.7
	"	24 " "	No study was possible since the blood hamolysed strongly.				
	0.003	2 " "	53.9	1.71	30.6
	"	24 " "	52.4	1.83	7.29	6.36	30.9
M. 4	Normal	60.3	1.48	7.31	6.45	29.5
	0.003	2 hours after	56.3	1.71	7.03	6.30	25.1
	"	24 " "	57.3	1.54	7.20	6.39	28.3
	0.01	2 " "	56.4	1.71	7.37	6.48	32.2
	"	24 " "	52.7	1.80	7.24	6.44	33.1

TABLE—*concl'd.*

Monkey number.	Dose per kilo in mg.	Time.	Surface tension.	Relative viscosity.	pH.	Buffer action.	Mobility $\times 10^6$.
M. 5	Normal	54.5	1.65	7.47	6.45	22.9
	0.01	2 hours after	53.1	1.71	7.20	6.67	21.8
	"	24 " "	51.7	1.71	7.47	6.45	20.0
	0.1	2 " "	50.8	1.80	7.26	6.65	26.2
	"	24 " "	52.9	1.67	7.30	6.37	23.3

ANALYSIS OF RESULTS AND DISCUSSION.

(a) *Surface tension and relative viscosity.*—It will be evident from the above that the surface tension invariably falls with the injection of the venom. The decrease is clearly evident even two hours after the injection. The second dose in all the cases had a cumulative effect. The case of monkey No. 1 is a little different in the sense that there is at first a fall in surface tension two hours after the introduction of the venom but there was a slight rise after twenty-four hours. Whether this indicates a slight tendency towards development of tolerance in this case is, however, not clear. The changes in all the cases were so small that it would not be justifiable to draw any conclusions from them. The maximum fall of surface tension was from 60.3 to 52.7 dynes in the case of monkey No. 4.

The changes in the relative viscosity of the plasma as compared to water at the same temperature are, however, more irregular. In most of the cases it has been observed that the immediate effect on viscosity is an increase which in many cases persists even up to twenty-four hours or gradually tends to come back to its normal value. When the second higher dose is injected the rise in viscosity after two hours is not as much as under similar conditions with the first dose.

From the above it is evident that both viscosity and surface tension suffer changes on injection of cobra venom but they come back or tend to come back to near about the normal value in about twenty-four hours' time. The directions in which these two factors alter are opposite to each other. It is known that surface tension varies inversely as the concentration of the proteins of the plasma while the viscosity bears a direct proportionality with this. Hence in the present case the diminution of surface tension and a simultaneous rise of viscosity indicates a probable rise in the concentration of the plasma proteins. This, however, suggests that it is the concentration of proteins in the plasma that is really changing and alterations in surface tension and viscosity are but outward manifestations of the same. Chopra and Chowhan (1934) showed that when the venom of Russell's viper was injected into cats there was an excessive leakage of blood fluids through capillary walls. The number of red cells was observed to be markedly increased in such cases immediately after the injection of a large dose of venom. The effect was not due to the induced polycythemia, but to the leakage of blood fluid which led to the concentration of the blood corpuscles. The protein may also in such

volunteer was available for a free gift of blood and no attempt was made in this direction.

This method was submitted to very rigid tests and was found quite satisfactory for accurate determination of lead in all biological materials. It consists in oxidizing urine (not less than 500 c.c.) and fæces (not less than 25 grammes) by means of nitric and sulphuric acids and then extracting the lead with a chloroform solution of diphenylthiocarbazone (dithizone). The extract is oxidized in the same way and treated with ammonia, ammonium acetate, sodium cyanide, and sodium sulphide, and a colorimetric determination is made in the usual way. The method as devised by Lynch *et al.* (*loc. cit.*) produces in many cases, especially in urine, a yellow coloration which sometimes becomes so deep that the solution becomes useless for matching and the experiment has to be abandoned. A slight modification casually suggested by the authors but not tried and confirmed by them has proved very satisfactory in our hands and in not a single case of this series has the disturbing factor spoilt any of our experiments. It consists in treating the oxidation products, before and after extraction with dithizone, with saturated ammonium oxalate solution and subsequent destruction of the oxalate with sulphuric acid.

It has been shown by Lynch and his co-workers that bone and teeth form the lead depôts in our system and gradual elimination of lead takes place through urine and fæces. We have found that the amount of lead eliminated through urine is not uniform but shows a wide fluctuation from day to day (*vide* Table IV), while the elimination through fæces is fairly constant and the amount of lead is much higher than in urine. It has been observed by Leschke (1934) that administration of sodium bicarbonate in heavy doses mobilizes lead from the bones to the general circulation and consequently larger amounts can be detected in blood, urine, and fæces. The examination of urine for diagnosis of lead-intoxication is not always a satisfactory procedure as no lead is found in urine if the kidneys are damaged. The examination of fæces or both urine and fæces is useful, especially if it is undertaken during a course of heavy doses of sodium bicarbonate.

In this investigation the samples of urine were collected for 24 hours, without adding any preservative, in a large Pyrex flask especially cleaned for this purpose and 500 c.c. were measured out for analysis. The fæces were collected from fresh morning specimens and were kept covered in a large Pyrex Petri dish and 25 grammes were taken for analysis. In every case, a duplicate experiment was performed. In the beginning of the investigation, the experiments were repeated with dry specimens of fæces (dried to constant weight) and it was found that the factor for conversion of the result from the wet to the dry state was fairly constant and was on an average 4.52. The figures given in the tables indicate lead content of fresh specimens of fæces. If they are, however, multiplied by the factor 4.52, the total lead content of dry fæces would be obtained.

Blanks with known weights of all the reagents and with the same glassware used in these experiments were repeated at frequent intervals and the amount of lead found in these reagents and glassware was quite small and constant with every new set of reagents. The blank figure was deducted from the figures obtained for urine and fæces and the actual lead content has been given in the tables.

TABLE I.
Normal and suspected cases.

Number.	Name.	Religion and sex.	Ago.	Occupation.	Mg. of lead in urine per litre.	Elimination of lead through urine (mg. per day).	Mg. of lead in faeces per kilo.	REMARKS.
A. Normal cases.								
1	H. D. G.	H. M.	34	Chemist	0.002	0.0023	0.133	..
2	K. B.	H. M.	48	„	0.003	0.0031	0.10	..
3	S. D.	H. M.	34	'Kaviraj'	0.012	0.014	0.08	..
4	R. C. S.	H. M.	28	Clerk	0.012	0.013
5	S. C. P.	H. M.	25	Tailor	0.008	0.0088	0.08	..
6	S. S. R.	H. M.	45	Doctor	0.008	0.0082	0.10	..
7	S. D.	H. F.	48	..	0.004	0.0039	0.08	Vegetarian.
8	S. K. U.	H. M.	33	Police	Nil	Nil	0.12	..
9	A. K. C.	H. M.	38	Motor driver	0.002	0.0019	0.10	..
10	N. C. D.	H. M.	36	Tailor	0.002	0.0021
11	S. C. S.	H. M.	38	Motor driver	0.006	0.0069	0.10	..
12	M. M. G.	H. M.	37	Clerk	0.008	0.0067	0.08	..
13	S. C. M.	H. M.	45	Police	0.004	0.0048
14	I. P. G.	H. F.	28	..	0.002	0.0019	0.14	..
15	K. C. B.	H. M.	27	Student	0.008	0.0085	0.14	..
16	S. C. D.	H. M.	26	„	0.008	0.0088	0.13	..
17	D. D. B.	H. M.	22	Clerk	0.012	0.0129	0.14	..
18	A. C. G.	H. M.	34	Chemist	0.006	0.0058
19	P. K. C.	H. M.	26	Zemindar	0.012	0.0147
20	B. K. G.	H. M.	31	Clerk	0.10	..
21	P. N. M.	H. M.	35	Chemist	0.012	0.013
22	S. M. D. G.	H. M.	31	„	0.012	0.0125
23	B. R. M.	H. M.	37	Lab. Asst.	0.016	0.016

TABLE I—*contd.*

Number.	Name.	Religion and sex.	Age.	Occupation.	Mg. of lead in urine per litre.	Elimination of lead through urine (mg. per day).	Mg. of lead in faeces per kilo.	REMARKS.
24	B. K. G.	H. M.	30	Reporter	0·016	0·0161	0·14	..
25	R. C. C.	H. M.	30	Doctor	0·012	0·013	0·12	..
26	K. B.	H. F.	19	..	0·013	0·0093	..	Died of arsenical poisoning.
27	M. K.	M. M.	30	Clerk	<i>Nil</i>	<i>Nil</i>
28	M. H.	M. M.	31	„	0·026	0·027
29	A. H.	M. M.	24	Student	0·042	0·042
30	K. H.	M. M.	26	„	0·004	0·0044
31	K. A.	M. M.	24	„	0·02	0·02	0·16	..
32	M. K.	M. M.	28	„	0·004	0·0043	0·14	..
33	M. L.	M. M.	26	„	0·012	0·0123	0·12	..
34	M. H.	M. M.	25	„	0·008	0·0094	0·16	..
35	A. K.	M. M.	26	„	0·004	0·004
36	M. I.	M. M.	27	„	0·008	0·0087	0·14	..
37	M. N. A.	M. M.	34	Doctor	0·012	0·013	0·12	..
38	I. S.	M. M.	40	Farmer	0·12	..
39	A. K.	M. M.	30	Student	0·023	0·023	0·14	..
40	M. J.	M. M.	25	„	0·022	0·024	0·10	..
41	M. S. M.	M. M.	40	‘Durwan’	0·026	0·028
42	N. A.	M. M.	32	Student	0·012	0·012	0·13	..
43	E. K.	M. M.	28	„	0·008	0·008
44	H. J. B.	A. I. M.	20	„	0·032	0·033	0·16	..
45	S. K. E.	A. I. M.	21	„	0·024	0·024
46	F. H. H.	A. I. M.	21	„	0·028	0·028	0·13	..
47	C. D. B.	A. I. M.	23	„	0·036	0·039	0·13	..
48	C. H. J.	A. I. M.	22	„	0·04	0·048

TABLE I—*contd.*

Number.	Name.	Religion and sex.	Age.	Occupation.	Mg. of lead in urine per litre.	Elimination of lead through urine (mg. per day).	Mg. of lead in feces per kilo.	REMARKS.
49	L. D. R.	A. I. M.	24	Student	0.024	0.025
50	M. C.	A. I. M.	22	„	0.03	0.03
51	J. C. M.	A. I. M.	42	Electrician	0.028	0.035	0.15	..
52	S. C. C.	A. I. M.	50	Optician	0.032	0.033	0.18	..
53	N. O.	A. I. M.	45	Merchant	0.036	0.041	0.17	..
B. <i>Suspected cases (press employees, etc.).</i>								
54	R. C. D.	H. M.	40	Compositor	0.06	0.066	2.40	..
55	H. C. D.	H. M.	48	„	0.364	0.374	2.28	Epigastric pain, constipation, and nerve disorders.
56	S. N. M.	H. M.	22	Student	0.03	0.03	1.07	Wasting of intrinsic muscles of hands with fine tremor.
57	M. M.	H. M.	24	Solderer	0.124	0.219	1.05	Blue line on gums.
58	N. G.	H. M.	53	Compositor	0.054	0.059
59	P. K. D.	H. M.	21	„	0.032 0.010	0.035 0.011	0.6 0.18	Blue line on gums. After treatment.
60	K. P. D.	H. M.	24	„	0.066	0.164	..	Blue line on gums and colic pain.
61	R. N. O.	H. M.	—	„	0.008	0.008	0.14	..
62	N. C. B.	H. M.	51	„	0.028	0.028	..	Irregular heart and low blood pressure.
63	R. N. D.	H. M.	50	„	0.024	0.027	..	Blue line and high blood pressure.
64	A. N. C.	H. M.	45	„	0.051	0.052	..	Neuritis in both hands.
65	R. L. G.	H. M.	50	Stereotypist	0.068	0.065	..	High blood pressure with albumin in urine.
66	B. B.	H. M.	—	Compositor	0.008	0.009	0.10	..
67	B.	M. M.	30	„	0.144 0.068	0.151 0.064	4.46 1.14	Blue line. During treatment:

TABLE I—concl'd.

Number.	Name.	Religion and sex.	Age.	Occupation.	Mg. of lead in urine per litre.	Elimination of lead through urine (mg. per day).	Mg. of lead in fæces per kilo.	REMARKS.
68	A. S.	M. M.	28	Compositor	0.012	0.011	0.71	Blue line.
69	A. R.	M. M.	40	Bookbinder in a press.	0.034	0.035	..	Epigastric pain and heart trouble.
					0.014	0.015	0.15	After treatment.
70	M. E. A.	M. M.	—	Compositor	0.004	0.003	..	Gastro-duodenal ulcer.
71	K. M.	A. I. M.	25	„	0.53	0.525	4.50	Blue line.
72	R.	A. I. M.	28	Lino-worker	0.384	0.414	2.85	Blue line.
					0.012	0.012	0.80	After treatment.

Note.—H. M.—Hindu male.

H. F.—Hindu female.

M. M.—Mohammedan male.

A. I. M.—Anglo-Indian male.

TABLE II.

Lead in normal urine and fæces (mg. per litre or kilo).

	URINE.			FÆCES.		
	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.
Hindus ..	<i>Nil</i>	0.016	0.008	0.08	0.14	0.11
Mohammedans ..	<i>Nil</i>	0.026	0.014	0.10	0.16	0.13
Anglo-Indians ..	0.024	0.040	0.031	0.13	0.18	0.15

TABLE III.

Lead in urine and faeces of printing press employees—some with definite signs of lead-poisoning (mg. per litre or kilo).

		Minimum.	Maximum.	Average.
Urine	..	0.004	0.53	0.107
Faeces	..	0.100	4.50	1.530

TABLE IV.

Daily variation in excretion of lead in urine and faeces (mg. per litre or kilo).

Case number.	1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.
<i>Urine.</i>							
57	0.103	0.238	0.102	0.122	..	0.089	0.121
60	0.063	0.165	0.152	0.111	..	0.077	0.103
<i>Faeces.</i>							
57	..	3.23	3.2	3.2	2.8	2.8	2.4
60	3.15	2.9	2.9

TABLE V.

Variation of lead content of urine according to nationalities (mg. per litre).

	Minimum.	Maximum.	Average.	Authority.
German ..	0.01	0.550	..	Litzner and Weyrauch (1933) quoted by Ross and Lucas (1935). Kehø <i>et al.</i> (1935) quoted by Ross and Lucas (1935). Francis, Harvey and Buchan (1929). Cooksey and Walton (1929).
American ..	0.04	0.080	0.05	
British ..	Nil	0.133	0.04	
Australian ..	0.02	0.050	0.04	

We have examined 53 normal cases, i.e., persons not having any reasonable chance of exposure to lead in performing their legitimate duties, and 19 suspected cases, i.e., persons employed in printing presses and factories where they are exposed to lead, and the result obtained so far is interesting. It will be found (*vide* Table II) that Hindus excrete the smallest amount of lead in urine and fæces, while Anglo-Indians excrete the largest amount and Mohammedans occupy an intermediate position. Boyd and Ganguly (1932) examined 36 samples of normal urine of Hindus, Mohammedans, and Europeans by the qualitative method of Fairhall and they obtained similar results. It appears that diet is possibly the determining factor for these differences and that there is a relationship between the lead content of the common foodstuffs taken by different communities and that of the human tissues and excreta. An investigation in this direction has just been taken up under the auspices of the Indian Research Fund Association.

SUMMARY.

The amount of lead eliminated by Indians in the fæces is about 10 times the amount in urine. In the case of the Anglo-Indians it is about 5 times. The average amount of lead in the urine of Indians is much less (about 1/3) than is found in the urine of Anglo-Indians which shows a close resemblance in this respect to that of the Europeans and Americans. Hindus and Mohammedans also show a difference in the amount of lead in urine and fæces.

For a chemical diagnosis of lead-poisoning, examination of both urine and fæces is useful.

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T. LENIA SOLIUM AND CYSTICERCUS CELLULOSÆ IN INDIA.

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IN the last few years considerably enhanced importance has been given to the question of human cysticercosis caused by *Cysticercosis cellulosa* on account of the work of several officers in the Royal Army Medical Corps whereby they have shown that systemic cysticercosis, especially in the case of brain involvement, is a fairly common and often fatal disease in British soldiers. They have also demonstrated that nearly all of the men so affected have served or are serving in India, and apparently acquired their infection here. The importance of this condition to India is thus clear, and as there is no apparent reason why British soldiers should be the only ones affected in this way it was considered worth while to try and discover from published records what evidence there is of the presence of *T. solium* and *C. cellulosa* among the indigenous population. The result of this search of the literature is summarized below. It is not claimed that the papers quoted give a complete record of all there is on the subject, but it is all that we have been able to find in the literature available, and as they include all of importance in the two principal medical journals of India as well as references to several English journals it is probable that not a great deal of valuable information has been missed. For the sake of simplicity the paper is divided into three parts, one dealing with *C. cellulosa*, two with *T. solium*, and the third a discussion of the problem so revealed.

Cysticercosis.

The editorial in the *Indian Medical Gazette* for November 1870 is devoted to a discussion on the importance of tapeworms in India with special reference to

'measly' pork and beef, and in this article we are taken back to the year 1844, for a report in that year on smallpox in Calcutta, by a Dr. Duncan Stewart, is quoted in which he refers to the frequency with which pork has to be thrown away on account of what is obviously cysticercus infection. The editorial proceeds to enlarge on this fact, and in the light of inquiries made in the year 1870 makes the following statement, which we quote at some length: 'The native pig slaughterers are quite familiar with the measles in pork and have a name for it—*dalim* or *dalimb*. It is never seen in home-fed pigs but in the mofussil pigs it is very common, especially those from certain districts; while pigs imported from other parts are entirely free from it. While evidence of cyst-infected pork is thus abundant, it is impossible to say to what extent tapeworm is caused by its consumption. Among 1,322 preparations which are described by Dr. Ewart in the catalogue of the museum of the Medical College there are only three specimens of *Tænia solium*, and two of these are without a head. In an article in the *Medical Times and Gazette* for 1857, *Tænia solium* is said to be not uncommon in India. It is plain then that country pork should be most studiously avoided; but if the feeding of pigs in India or elsewhere is properly conducted there is little fear of contracting tapeworm.'

The interest of this quotation at the present day lies in the fact that, as far as the writers have been able to ascertain, they apply to conditions in Calcutta to-day as well as they did on the day the article was written, in spite of the great improvement in preventive medicine.

The first record of human cysticercosis appears to be the report by Armstrong (1888) of the finding of *C. cellulosæ* in the brain and heart of a criminal lunatic who died in Nellore gaol in 1887, and in the same report he remarked that this infection is said to be very common in Madras. The next record is that of Williams (1906) who removed a cyst from the tongue of a prisoner in Coimbatore gaol. This was identified as *C. cellulosæ*, and he mentions in the same paper that he found the same parasite in the brain of another prisoner who died of pneumonia. Elliot and Ingram (1911) removed a cyst of *C. cellulosæ* from the eye of a French Eurasian from Pondicherry, and in the same paper they call attention to a post-mortem on a Hindu male, previously recorded in the Annual Report of the Madras Hospital for 1910; in which numerous cysts were found in the brain. A heavy infection of the brain and skeletal muscles was reported by Trimurti (1911) in another Hindu admitted to the Madras Hospital with a history of sudden onset of paralysis and who died two days later with symptoms resembling those of meningitis. Campbell and Thompson (1912) saw a similar heavy infection in a Hindu in Madras Presidency. The next reference we can find is that by Wright (1923) who removed a sub-conjunctival cyst, also in Madras. This was subsequently identified by Leiper as *C. cellulosæ*. Two years later, Ware (1925) recorded two cases of cysticercus infection in the eye and one in the brain in the same locality.

These ten cases are all that can be found in the literature which covers a period of 59 years.

Since the importance of this condition has attracted the attention of pathologists in the Royal Army Medical Corps a good deal of work has been done on the subject and Dixon and Smithers (1934) give a summary of the cases they were able to collect in the records of the British army and they list 71, of

which number 64 had been in India shortly before or at the time they first showed signs of cerebral involvement.* In only 17 of these 64 cases is there any history of an adult worm being present; three of these were identified as *T. solium*, one as *T. saginata*, and the remainder was unidentified. In a final valuable summary of the position in which the question is discussed in every aspect the same authors, Dixon and Smithers (1935*a* and *b*), add another eight cases to the above. All of them had been in India and in four the adult worms as well as cysticerci were found, these were identified as follows: 1 *saginata*, 1 *solium*, 1 not identified, and 1 identified on two separate occasions as *solium* and *saginata* respectively. This brings the total number recorded by these workers to 72 in which 21 had adult tapeworms as well. Since that time Lipscombe (1935) and Dogra and Ahern (1935) have recorded two more cases in British soldiers, both of whom were in India. Lipscombe's case passed a *T. solium* after treatment. To these might be added eight cases reported by Lindeman and Lyburn (1935) in Khartoum, for they found, in a regiment recently transferred from India, two cases with fits and cysticerci, two cases with cysticerci with no symptoms, and four cases with suggestive nervous signs but no demonstrable cysticerci. This gives a grand total of 82 cases in 22 of which there were also adult tapeworms.

The first case in Dixon and Smithers' list was recorded in 1892 and before the next there is a gap of about 22 years so that 81 cases have been reported over a period of about 23 years in British soldiers who had served in India. This is in striking contrast to the ten cases in 59 years recorded from the indigenous population.

Morrison (1936) has found ample evidence of pig infection with *C. cellulosæ* in South India.

Tænia solium.

Although it is clear from the editorial in the *Indian Medical Gazette*, already referred to, that tapeworm infection with both *T. solium* and *T. saginata* must be common in India no special surveys have been made to ascertain the incidence of these infections nor to distinguish between them so that any early references to the subject in Indian medical literature are only vague remarks regarding the presence or absence of *Tænia* in various parts of the country.

In the last twenty-five years, surveys, with the object of finding the incidence of helminths, have been undertaken, but most of these had as their primary object the investigation of hookworm prevalence so not much attention was paid to other worms. A certain number of the workers have noted the other worms or their eggs that were encountered in the course of their surveys, and these records are all that is available on the subject of tapeworm in India.

The first survey of this nature that we are able to find is that of Lane (1909) who recorded finding the ova in five and the proglottides in fifteen of the prisoners in Monghyr gaol, this gave an incidence in the gaol population of 3.75 per cent. All of these infections were said to be *T. solium*. The next report is that by Sinton and Baily (1916) who, without giving figures, say that *T. saginata* is sometimes

* The references of this series are given both in the paper quoted here and also in Dixon and Smithers (1935*b*) so they are not repeated in this paper.

found in Kohat but that *T. solium* is unknown. Mhaskar (1917, 1919) in the coolie emigration depôt at Negapatam found 30 *T. solium* and 24 *T. saginata* out of 5,882 examinations, and in the town itself he found 11 *solium* and 13 *saginata*; in Trichinopoly gaol there were 1 *solium* and 1 *saginata* out of 612, while in Dindigul town he found 4 *solium* and 2 *saginata* out of 412. All these cases of Mhaskar's appear to have been diagnosed by recognition of eggs in the stools and not by examination of segments. It should also be noted that as Mhaskar was primarily concerned with estimating the hookworm infection rate any given stool examination ceased when a hookworm egg was found, so it is probable that some tapeworm cases were missed. Korke (1926) recorded one case of *saginata* in Singhbhum and 6 in Bihar; here again it is probably only eggs that were seen. Amongst the troops at Dehra Dun Covell (1926) discovered 50 *Tænia* infections out of 1,188 stools examined using the 'D.C.F.' method. As *Tænia* eggs do not usually rise to the surface by this technique and *Hymenolepis* eggs do, it seems doubtful if any of Covell's cases was even *Tænia*, quite apart from which species of the latter was involved.

Chandler (1926-1928), in his somewhat sketchy survey of the whole of India, came to the conclusion that *Tænia* infections were practically non-existent among Bengalees, but that in Northern Bengal they are very common among the hill people, who are in the habit of eating raw or improperly cooked beef and pork, so it is probable that both *T. solium* and *T. saginata* are present. In the examination of 12 'random' specimens from Kalimpong, however, he only found the latter species. In Assam no *Tænia* were found and in Burma the infection was confined to the Shans, 5 per cent of whom were positive; unfortunately no diagnosis was made of the species harboured. In Bihar he found 2 *Tænia* out of 1,020 and in the United Provinces 6 out of 823 stools examined. Only two cases were found in the whole of North-Western India, including the extensive area of the Punjab right up to the Frontier. In Central India and Bombay he found only three cases, all in the vicinity of Jubbulpore. In Madras and South India Chandler found 1 *Tænia* infection in 1,603 stools examined. He quotes figures given him by Kendrick who found approximately 122* *Tænia* infections out of a total of 34,817 examinations, and because he himself found 3 per cent of *H. nana* infections in his small series assumes that most of Kendrick's cases are of the latter species and not *Tænia* at all. This assumption appears unjustifiable especially as Mhaskar, working in the same part of India, recorded several *Tænia* infections but did not mention *Hymenolepis*.

This is as far as the published records we have been able to find take us, and it must be admitted that when the immense population of India is considered these figures are so small as to be useless in arriving at an estimate of the incidence of *Tænia* infection. From the point of view of the present inquiry, which only has *T. solium* under consideration, the information is still more unsatisfactory, for it is doubtful if any of the records can be regarded as definitely indicative of *T. solium* because they have nearly all been made from egg examinations only, and it has been shown by Maplestone (1937) that differentiation of the eggs of

* The figures are given in Kendrick's table in percentages with a decimal point so the figure given is only approximate as the actual number of infections found in each series could not always be determined.

T. solium and *T. saginata* is not possible. The only instance where proglottides are mentioned is in the case of Lane (*loc. cit.*), but he makes no special mention of having examined the segments and also refers to eggs in five of his cases, so it is possible that no definite diagnosis was attempted. Chandler apparently examined 12 worms from a locality where it might have been anticipated *T. solium* would be found, and all of them were *T. saginata*.

Since the Calcutta School of Tropical Medicine has been opened all the tapeworms recovered have been carefully examined and the species determined. Out of 137 cases treated only one *T. solium* infection has been found, and this surprisingly in a Mohammedan. The writers have two other unpublished records of *T. solium* that they have examined. The first of these was a worm sent from Lucknow; it had been removed by treatment from a British soldier who was considered to have contracted the infection in that station, and the second was found in a girl in Puri district under the following circumstances:—

Records of the district of origin of cysticercus infected pig was obtained from the Calcutta slaughter-house and a number were reported to have come from the Puri district, where it was possible to actually determine the villages the pigs came from. A visit was paid to the area and a few stools were collected from the inhabitants who were pig-breeders. Sixty-four stools were collected and in one passed by a girl undoubted segments of *T. solium* were found. It was difficult to carry out even this small investigation because of the ignorance and suspicion of the villagers, and for this reason treatment was refused by the infected person, so the whole worm was not recovered. Also further work could not be carried out at this time for want of personnel and funds to conduct a more extensive survey.

It is probable that more definite information will soon be available from army records regarding the incidence of *T. solium* among British troops, for it is believed that in future all tapeworms have to be identified [Dixon, in Dixon and Smithers (1935a), records 1 *T. solium* in 50 tapeworm cases at Poona in British soldiers during the years 1928 to 1932]. This information, when it is made available, will certainly be of value but it will not touch on the root of the trouble. This will not occur until records from the indigenous population are collected.

DISCUSSION.

The above summary of the available information regarding *C. cellulosa* and *T. solium* in India presents several peculiarities that appear worth consideration.

The first of these is the apparent rarity of *T. solium* in the indigenous population and the relative frequency of *C. cellulosa* in pigs slaughtered in various parts of the country. Man is the only known host of the adult worm, so unless some other not yet recognized host of this worm exists (a most improbable contingency) it must be accepted that *T. solium* is more common in India than present records indicate. This contention is, to some extent borne out by the rapidity with which one case of *T. solium* infection was found when it was looked for in a probably favourable locality, indicated by working back from infected pigs slaughtered in Calcutta to the place where they were bred. Our ignorance on this point can be readily explained by the fact that nearly all the people in India who have to do with pigs are of low caste and illiterate, and they live in

primitive fashion in places remote from centres where skilled medical attention is available, so it is conceivable that a fair proportion of them harbours tapeworms. These worms as a rule cause little disturbance to health, so it is possible their presence is ignored by their ignorant hosts, and hence no record of their presence gets into medical and public health returns.

The matter of cysticercosis is, however, somewhat different because it seems unlikely that even these low-caste people would allow their relatives to die without going to considerable trouble to obtain medical aid for what must appear to them as a mysterious and terrifying disease as it frequently gives rise to fits and mental derangement for prolonged periods before death occurs. On this account it seems unlikely that the paucity of records in hospital returns of cysticercosis is altogether misleading, and it is considered probable that this condition is not very common in Indians. On the other hand, the experience of the military authorities who, since they began to look for it, have found 81 cases in about 23 years among the comparatively small numbers of the British army in India in contrast to the immense indigenous population suggests that the disease must be more common than we realize.

One possible explanation of the apparent difference in the incidence of cysticercosis in British soldiers in India and its native-born inhabitants being real and not due to lack of information suggests itself to us. There may be a relative immunity or at least enhanced resistance to infection in the local inhabitants, especially in those who have been living in close contact with pigs for many generations and under very insanitary conditions so that the opportunities for infection with both *T. solium* and *C. cellulosæ* were always present, and that this state of affairs has lasted so many years that resistance to infection with the larval stage is now transmitted hereditarily. British soldiers, many of whom seem to acquire this infection soon after their arrival in India, would not be expected to have any acquired or transmitted resistance to helminth infections, and would consequently be much more readily infected if the opportunity arose. There is no evidence at present that such immunity can occur in the case of *C. cellulosæ*, but the possibility is suggested by the work of Penfold and Phillips (1936) who have shown that immunity can apparently be acquired to an infection by *C. bovis* in the case of calves. This is of course a long way from showing that resistance can be transmitted but it is at least suggestive of the possibility.

Another unusual feature in the case of the soldiers is that out of 82 recorded cases evidence of previous infection, or infection at the time they were found to be suffering from cysticercosis, with an adult worm was only obtained in 22. It has been suggested on good authority and as the result of personal experience of a well-known helminthologist in England, that autodigestion of mature segments occurs so that an infection may persist for years without any evidence of its presence being manifest in the stools. This in the one instance referred to is undoubtedly true but it seems improbable that it can be common enough to explain the fact that at least 73 per cent of infected soldiers had unrecognized *Tænia* infections and acquired their cysticercosis by autoinfection. As opposed to the above single instance of prolonged digestion of tapeworm segments, our experience in two cases of experimental infection with *T. solium* may be quoted. Both of these cases began to pass segments about three months after being given cysticerci and they passed segments daily as long as the infection was allowed to remain,

though it must be admitted this was not longer than a fortnight on account of the danger of cysticercosis being acquired by these persons. It is accordingly suggested that it seems likely that most of the soldiers acquired their infections in the normal manner, that is by swallowing eggs and not by eggs from worms in their own intestines, although it must be admitted that no explanation is at present forthcoming as how these eggs came to be swallowed.

It is also surprising to note that Dixon and Smithers (1935a) consider most of the soldiers became infected in Northern India whereas all the local records of cysticercosis in the indigenous population come from Madras Presidency.

This brief survey of the puzzles in the epidemiology of *T. solium* and cysticercosis in India suggests two possibilities: one that there are factors in the transmission of these infections with which we are at present not acquainted, and that the accepted life-history of this worm is subject to certain modifications depending on local conditions, and the other is that our records are at present altogether misleading. Whichever of these be accepted it is clear that there is a great deal to be known about the epidemiology of this parasite in India before the disease can be adequately controlled.

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part I.

INTRODUCTORY NOTES AND A HISTORICAL SURVEY.

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THE outline of the epidemiology of most of our common diseases has already been firmly drawn and a great many details have been filled in, though the picture cannot be claimed, in many cases, to have yet been completed. With epidemic dropsy we are in a much less happy position. Since the disease first attracted attention fifty-eight years ago, quite a volume of literature has accumulated on the subject, but epidemic dropsy cannot be listed amongst the conquered diseases. When, therefore, towards the beginning of 1935, the district of Manbhum, Bihar, was in the grip of a raging epidemic of dropsy and the administrators looked for basic knowledge on which to formulate their relief measures, the Calcutta School of Tropical Medicine and the All-India Institute of Hygiene and Public Health jointly organized an inquiry under four different sections, viz., (i) clinical and experimental, (ii) epidemiological, (iii) bacteriological, and (iv) biochemical. In this and subsequent communications we propose to deal mainly with the epidemiological aspects of the inquiry.

HISTORICAL.

According to the available records a 'new disease' which we can now recognize as epidemic dropsy first made its appearance in Calcutta in 1877 (Smith, 1880),

though Chambers (1880) would have us believe that 'the disease has been prevalent in India since the famine of 1866'. Cayley (1878) was the first to describe the clinical signs and symptoms of eight cases which resembled epidemic dropsy in all respects, but the swelling of the upper extremities and the face seems to have been more common in his cases than what is usually observed in recent epidemics. Payne (1879), in his report on the health of Calcutta for the 3rd quarter of 1878, also alluded to this new disease. McConnell (1879) also referred to this recurrence of the disease in November 1878. Its leading features were fever, anæmia, exhaustion, and œdema of the feet and ankles and also of the upper limbs, the face, and the neck, and in rare instances of the whole body. He ascribed the symptoms to the attack of malaria in persons of low vitality due to lack of proper nourishment. The dropsy, he thought, was due to the effect of cold and damp during fever, excessive rains contributing towards these conditions. Crombie (1879) reported the prevalence of 'Acute Œdema in Dacca', some months after it had started in Calcutta. He noted that he had seen an exactly similar case at a lecture demonstration in the Royal Infirmary at Edinburgh in 1866. O'Brien (1879a) reported 12 cases of 'Acute Œdema' and thought that the disease was due to lateness of rains and consequent excessive humidity affecting a population with a scorbutic diathesis and suffering from chronic malnutrition. However, in May, the same year, this investigator (O'Brien, 1879b), having studied 200 cases in Shillong, abandoned his previous notions of the ætiology of the disease in favour of 'contagion theory' basing his opinion on the following observations, viz. :—

- (1) the disease broke out as a widespread epidemic in parts of Assam, shortly after its appearance in Calcutta and Dacca,
- (2) it attacked the entire households and from there spread to the houses of friends visiting them. Concrete examples of such extensions were cited,
- (3) the climate of Shillong was dry and warm at the time of the outbreak. The association of scurvy and dropsy was accidental.

Thus O'Brien was the first to advance the contagion theory of the ætiology of epidemic dropsy.

A society of leading medical men, called the 'Calcutta Medical Society'; was established early in 1880 and some very interesting observations regarding epidemic dropsy were made in its monthly meetings (*Indian Medical Gazette*, 1880). The Editor, *Indian Medical Gazette* (1880), remarked that 'even as regards the question of infectiousness, it is impossible as yet to pronounce a decided opinion. It is certainly a spreading disease; but the spread is slow, continuous, and affected by seasonal conditions. It also affects groups of people, households, whom it attacks simultaneously, or in rapid succession; but whether the cause is communicable or common it is not easy to say'. Deakin (1880), from an investigation of an outbreak in the Allahabad jail, thought that this was due to contamination of the water-supply from a particular well. Khastagir (1880), on the other hand, thought that the 'new disease' was of an infective nature and all the mucous surfaces of patients were affected. The epidemics of 1878 to 1880 in Calcutta, Shillong, and Mauritius were admirably reviewed by McLeod (1893), to whom we owe the present name of this 'new disease'. He concluded that the malady was communicable from person to person and portable by human agency, but its

diffusion was feeble and depended on seasonal and other conditions which modified its epidemic vitality and determined its transmission and extension. He further held that 'the unit group was the family or household, and the composite group the community of families of the same race, caste, or occupation and habits associating in domestic intercourse, work, workshop, etc.; this grouping furnished the key to diffusion and progress of the epidemic. Lovell (1881), however, claimed that the epidemic at Mauritius in 1879 was not contagious.

For some years following McLeod's observations, reports were received from various places in Bengal and Assam about epidemics of dropsy. Rogers (1902) reported a mild recurrence of the epidemic in Calcutta in 1901 and Cobb (1903) described an outbreak in the Barisal jail which he thought was due to infection through the intestine. Deaths from this disease continued to be recorded (Mazumdar, 1933) in Calcutta every year during 1905 to 1931 with occasional exacerbations. Cases were also reported from Howrah, Kurseong, Mymensingh, Chittagong, Sylhet, Gauhati, and Shillong (McLeod, 1909). Daley (1908) reported fifty cases in the Reformatory School at Alipore. S. Anderson (1908) reported an outbreak in the Comilla jail, where he found a remarkable grouping of cases and concluded that the disease was of the nature of an exanthem.

Munro's (1908) report on the outbreaks in Darjeeling district is interesting and serves as an introduction to the present-day theories of the ætiology of epidemic dropsy. Discarding his previous conception of place infection, he discussed the following possibilities :—

- (i) an infection of bacterial or parasitic origin,
- (ii) a disorder of nutrition, or
- (iii) a chronic food intoxication.

By a process of elimination, he concluded in favour of the last-named hypothesis.

As regards the specific offending article of diet he argued that it should be common to both the epidemics investigated by him and that it should be one consumed in large amounts. Rice appeared to him to be the most likely agent though he thought *dals* should not be dismissed from consideration. He further observed that the disease appeared during or after the rains when local rice was scarce and imported rice was mostly used. In order to explain his observation that sometimes only a single member of the household was affected he contended that all sources of supply were not likely to be poisoned and even in a particular stock the poisonous elements were not evenly distributed. Moreover, the resistance of different individuals to the disease varied greatly. It is in Munro's report that we find the first reference to mustard oil as a causal agent. The author himself did not believe it to be so, but he simply mentioned it as a popular belief with the laity. According to Basu (1935), Mitra, as a result of his investigations at Howrah, was the first physician to incriminate mustard oil as the causal agent. Delany (1908), who made investigations into the causation of epidemic dropsy or beri-beri in Eastern Bengal, advanced a theory that epidemic dropsy was a specific, infectious or bacterial disease and that it was conveyed from person to person by bed bugs.

Lukis (1908) made some very interesting clinical observations in Calcutta. He came to the conclusion that 'epidemic dropsy is of the nature of an angio-neurotic

œdema and that it is closely allied to such diseases as urticaria, erythema nodosum, and the like. If then, epidemic dropsy is allied to the urticarias it is probably due to some form of sepsis'. Against the theory that epidemic dropsy was a form of beri-beri, he put forward two arguments, viz.:—

- (1) complete absence of paralysis or anæsthesia, and the exaggerated knee-jerk,
- (2) 'the fact that although beri-beri has been for years endemic in the Chinese quarters, there is no sign of any increase of the disease in that locality. On the contrary, during the past year fewer cases of beri-beri have been admitted into the Chinese ward of the Medical College Hospitals than has been the case in the former years, and I have never seen in a Chinaman any case resembling in the remotest degree the epidemic dropsy which is now prevalent'.

Campbell (1908) reported a rather severe outbreak in the Dacca Lunatic Asylum where 155 out of 270 persons were attacked with the disease, giving a case incidence of 57.4 per cent. The author discussed various possibilities regarding the causation of the outbreak and produced strong evidence against the disease being communicable from man to man. The facts on which he based his conclusions were:—

- (i) that very few inmates were permitted outside the Asylum,
- (ii) that males and females lived in entirely separate blocks and there were no means of communication between them and yet the disease broke out equally on the same day in both parts,
- (iii) that of the 17 dangerous lunatics confined in entirely separate cells, 15 were affected during the outbreak,
- (iv) that no member of the staff nor visitor contracted the disease, and

The author then concluded that rice consumed by the lunatics was probably the cause of the epidemic, though he thought of fish also as a possible source of the trouble.

The year 1909 saw a moderately big epidemic of dropsy in Calcutta when 433 deaths due to this disease were recorded. At Howrah also it attracted serious attention of the medical practitioners as well as of the Government. Sen (1909), alluding to the disease then prevalent, made the following observations, 'many of us have seen the awful miseries the disease is causing in affected families, for we have never seen a more widespread disease than this. When it attacks a family, almost all the members are affected. We have seen epidemics of cholera, small-pox, and plague, but I think I am not exaggerating a bit when I say that, all these are more merciful and leave a larger number of members free than this disease, in any affected family'. He attributed the disease to the consumption of mustard oil adulterated with some mineral oil. Ghosh (1910), on the other hand, rejected this hypothesis and argued that epidemic dropsy was due to some infection through food, probably rice.

About this time, Megaw (1910) interested himself in the problem of what he called 'epidemic dropsy type of beri-beri' and published his first paper on the subject. Further reference to his work will be found below. Greig (1911, 1912) made a detailed investigation on the outbreak of epidemic dropsy in Calcutta in

1909 and concluded that it resulted not from infection or intoxication, but from the habitual consumption of unbalanced diet. He laid special emphasis on sustained deficiency of organic phosphorus in the food of races which were subject to epidemic dropsy and produced epidemiological, biochemical, and experimental evidence in support of his theory. He ascribed sharp outbreaks to high prices of foodstuffs which caused sudden upsetting of balance in the diet of the people who ordinarily lived on the margin of starvation. Greig's objection to the infection theory was based on the following observations, viz.:—

- (1) of 33 households in which cases of epidemic dropsy were imported, further cases developed only in two.
- (2) of 404 houses with a history of epidemic dropsy, in 163 instances single cases were reported and of these only 79 proved to be epidemic dropsy. Most of these cases were imported. Multiple cases in the family were the rule. From this he argued that the diet was to blame.
- (3) Megaw inoculated himself with 3 c.c. of blood from a case of epidemic dropsy without any ill effects.
- (4) in wards Nos. 5 and 7 where the disease was rife and where the Marwari population was predominant, epidemic dropsy was confined to the Bengalees and claimed no victims amongst the Marwaris.
- (5) cases have been treated in open wards without giving rise to secondary cases.
- (6) blood, stool, etc., proved negative on examination.

Greig, like Munro, found that the lay public blamed mustard oil, which they believed was adulterated with some unknown mineral oil. He ruled this out as an ætiological agent, basing his arguments on the following observations:—

- (i) 'bloomless oil', a sort of mineral oil, which was especially blamed was added to the food of some pigeons, but no deleterious effects were noted.
- (ii) an epidemic of dropsy broke out in the Busti jail in U. P. where only jail-made mustard oil was issued to the prisoners and 'it was not at all likely that adulteration with petroleum could occur'.

Greig's investigations attracted very wide attention and the Editor of the *Journal of the American Medical Association* (1911), noting the complete immunity of the Marwaris, remarked that 'such a distinction might of course argue for racial susceptibility, were it not for the fact that Eurasians and poor Europeans at times contract the disease and that a much more obvious and probable explanation is forthcoming'.

Lyngdoh (1912) gave an account of the occurrence of epidemic dropsy in Habiganj (Assam) and contended that the disease was communicable from person to person and no foodstuff played any part in its causation. Young (1912), on the other hand, thought that epidemic dropsy was infective in nature and spread through food like enteric fever or probably it was contagious like the specific fevers of Europe. He further suggested that infection was conveyed by early cases only.

In 1919 and 1920 there was a moderate outbreak of epidemic dropsy in Calcutta during which 208 deaths were recorded. Outbreaks of the disease were also reported from the interior districts of Bengal. Acton's (1922) previous work on lathyrism suggested to him the possibility of toxic amines being responsible for the causation of epidemic dropsy. He worked on Greig's data previously referred to and stated that 'it is with regard to his interpretation of the data that I differ from him, as I believe he was led astray by the vitamin theory then recently published by Fraser and Stanton'. He concluded by saying that 'speculations as to the nature of the toxin, etc., are premature but the evidence is definite that it is found in over-seasoned rice'. This was the first step towards the development of the classical rice theory of Acton and Chopra. Acton's observations appeared to be so striking and definite that they aroused considerable enthusiasm and led the Editor of the *Indian Medical Gazette* (1922) to announce that the problem of epidemic dropsy in Bengal had been solved. Bose (1924), however, disputed these claims. He was able to grow the spore-forming bacteria in the agar media from the so-called 'diseased' grains, as well as from the grains of rice which did not show white streaks when put in contact with water, and from the common market rice in Calcutta. On adopting a more efficient method of sterilization, he repeatedly failed to grow those organisms on culture, and therefore concluded that the spore-forming bacteria referred to were only external contaminations. These important observations do not seem to have attracted the attention that they deserved. Megaw (1923) discussed the 'Beri-beri and Epidemic Dropsy Problem' under three headings, viz.:—

- (1) Is epidemic dropsy a form of beri-beri?
- (2) Is avian polyneuritis the same disease as beri-beri?
- (3) What is the probable cause of beri-beri?

In discussing the first question, he put forward a vigorous plea as to why these two so-called different diseases should be considered as extreme types of one and the same disease. The two diseases, he thought, could not be distinguished from one another, and he pictured 'these extreme types' as standing 'at the ends of a chain of which every link is complete'. He preferred to group them together under the old name beri-beri, though he did not object to the use of such terms as 'epidemic dropsy form of beri-beri' or 'ship beri-beri'.

He based the answer to his second question on a comparison between the post-mortem findings in experimentally produced *polyneuritis columbarum* and those in human beings dying of beri-beri and came to the conclusion that the former disease 'which is caused by a deficiency of vitamin B has not been proved to be the same disease as beri-beri'.

With regard to the third question he said that 'the known facts suggest strongly that a poison formed in rice under certain conditions of storage may be the essential cause of some forms of beri-beri, and probably of the disease in general'.

Regarding mustard oil as the causal agent of epidemic dropsy, the author stated, 'to my mind the most unanswerable objection to the view is that outbreaks of a similar form of disease in other parts of the world occur among people who do not employ mustard oil or any of the suspected adulterants of that oil in their diet'.

Megaw's objection to the existence of a specific infection conveyed from man to man may be summarized as follows:—

- (1) No microbe had been shown to be uniformly associated with the presence of the disease.
- (2) Animal inoculation and feeding experiments had failed.
- (3) Serum from a patient injected into the author's arm had failed to produce any symptoms.
- (4) Quick response, in the early stages, to change of diet. If it were an infection it should be expected to run its course.
- (5) Persons brought into intimate contact with patients were not more liable to contract the disease than others living on the same diet.
- (6) Management of outbreaks on the assumption of their being due to infection had always failed.

On the other hand, Brahmachari (1923), as a result of his investigations at Krishnagar and some villages in Bengal, was inclined to believe in the infectious nature of the disease. K. C. Dutt (1924), on the basis of his observations on 1,700 cases, was convinced that the disease was a manifestation of intestinal infection with a specific micro-organism, though none could till then be isolated and demonstrated.

The year 1925 is the most important landmark in the development of our present conception regarding the ætiology of epidemic dropsy when Acton and Chopra (1925), as a result of extensive laboratory investigations, enunciated their famous 'rice theory'. This theory is too well known to require a detailed description and it will suffice to give its barest outlines.

It states that some varieties of rice, when stored under suitable conditions of temperature and moisture, are liable to get infected with certain organisms belonging to the *Bacillus vulgatus* group. These organisms produce a water-soluble toxin in rice, which, when ingested, gives rise to epidemic dropsy. Parboiling kills the enzyme and over-polishing, which occurs in the process of milling, damages the grain, thereby rendering it easier for the organisms to invade the rice. As a result of such an invasion, the central part of the grain is rendered opaque. A different variety of rice, under similar conditions of storage, gives rise to an alcohol-soluble toxin which produces symptoms of beri-beri.

The laboratory evidence in support of the theory rested on (1) bacteriological examination of the rice, (2) isolation of the toxin, (3) pharmacological action of the isolated toxin, and (4) feeding experiments on monkeys.

The experimental evidence produced by these authors appeared to be so complete and convincing that the 'rice theory' became more or less universally accepted.

A discordant note was, however, struck by I. R. Anderson (1927). He investigated an outbreak amongst a semi-isolated community at Kalna, Bengal. By carefully following the distribution of cases and the dates of purchase and sources of provisions he was able to exclude rice as an ætiological agent. The outbreak followed the importation of five cases of epidemic dropsy and the author produced very strong evidence to support the 'contagion theory'. Rice theory

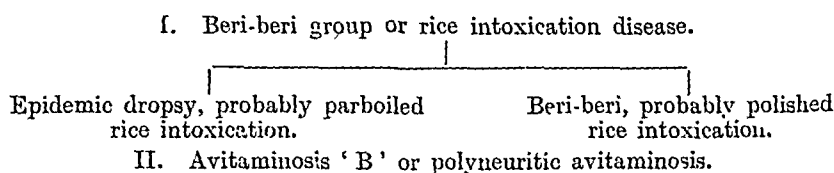
being then in vogue he directed his attention solely to this article of food and unfortunately did not take other food articles into consideration. ...

Commenting on Anderson's paper, the Editor, *Indian Medical Gazette* (1927), argued that facts brought out in that communication strongly favoured the possibility of a poison being produced in the intestinal tracts of persons who had become infected with a special organism, but he pointed out that this mechanism of disease production was not at all common. As a general rule, the observed facts could best be explained on the hypothesis of intoxication due to a pre-formed toxin in rice, but it was quite possible that in exceptional cases the poison might be formed by microbic action on the intestinal contents.

Calcutta again experienced a severe outbreak of epidemic dropsy in 1926-27 when 1,575 deaths occurred. The medical practitioners discussed the problem at a conference (Calcutta Medical Club, 1926) from different aspects, but no definite conclusions could be arrived at. In the following year, Acton and Chopra (1927) confirmed their previous findings. However, Gloster (1928) was unable to confirm the bacteriological findings of these authors.

The Far Eastern Association of Tropical Medicine held its session in Calcutta in December 1927 and the problem of epidemic dropsy and beri-beri received its due share in the deliberations. Megaw (1927) who opened the discussion stated that beri-beri could be caused by two factors, viz., (1) rice intoxication, and/or (2) 'avitaminosis 'B'.

He proposed the following classifications :—



Megaw thus postulated two doors of entry for the disease, one through pre-formed toxin in rice and the other through dietetic deficiency. In the discussion that followed, various theories were advanced and refuted. Thus Bose attacked mustard-oil theory on the ground that cases of epidemic dropsy had occurred amongst the Burmese who never used any mustard oil, but used gingelly oil.

Megaw *et al.* (1928) continued their studies on the 'epidemic dropsy form of beri-beri' and made some very interesting field investigations concerning the ætiology of the disease. They stated that 'it is impossible' to reconcile every reported circumstance with any one hypothesis'. However, with the help of maps of the affected localities they showed that 'the disease almost always selects persons who have been using a stock of rice from a common source'. They further stated that 'most of the outbreaks which have been described in this paper can only be explained on the hypothesis that the rice in use by the patients had in some way become poisoned during the period of storage, yet there are some observations which if taken by themselves would point to other possibilities, notably mustard-oil poisoning and infection'.

Kamath (1928) referred to an outbreak in a village in the Madras Presidency where all the victims used gingelly oil only: His observations of the outbreaks

amongst the inmates of a Mission House where five out of six Europeans were affected, as also amongst the clients of a hotel, were striking in many points. He claimed definitely to exclude rice as the causal agent, at least so far as the Mission House cases were concerned. From the evidence collected, person-to-person spread of the disease also seemed to him extremely unlikely. He showed that there were two sources to which more than 75 per cent of his cases could be traced. The mode of spread, he thought, was through food which had suffered contamination of human origin.

On the other hand, Sagayam (1927) reported an outbreak of epidemic dropsy amongst Indians in Fiji and produced fairly strong evidence in support of the mustard-oil theory. Rice he excluded on the ground that while the Chinese, Fijians, and Indians consumed rice from the same stock, the disease was confined to the Indians. The Editor of the *Journal (Indian Medical Gazette)* did not find Sagayam's arguments convincing and commented that the observations made by the author were not inconsistent with the view that the outbreak was due to importation of an infected supply of rice from Calcutta during 1926 when Calcutta was subject to a rather severe outbreak of epidemic dropsy. However Banerji (1929), who had investigated a small outbreak in Bengal, came to the same conclusion as that arrived at by Sagayam.

An outbreak of the disease at Allahabad was investigated by Banerjee (1928), an old collaborator of Megaw. He had to revise his old notions on account of certain interesting observations made by him in the course of these investigations. There were altogether about 200 cases of the disease, all but six of which occurred amongst the Bengalees. One hundred out of the 500 Bengalee families were affected. In three of the affected families rice had been altogether given up for nearly a month before they were attacked. Another interesting happening came to his notice which further created doubt in his mind about the validity of rice theory. Seven Bengalee Hindu families and four local Mohammedan families happened to have purchased more than a month's supply of rice at the same time from the same stock. Five of the Bengalee families were stricken, while all the Mohammedan families escaped. However, this argument would appear to lose much of its force when it is remembered that the U. P. Mohammedan does not, as a rule, consume rice to the same extent as an average Bengalee does. Moreover, the common supply of rice was obtained two months previous to the outbreak of the disease and the probability of the Bengalee families having purchased rice, which was responsible for their troubles, at a later date, could not be eliminated.

Dalal (1929) gave an interesting account of an epidemic at Rangoon in July 1924. But for two cases who were Upper-India Hindus, all his 174 cases occurred amongst Bengalee Hindus. He suggested that the outbreak was due to a particular consignment of 'diseased' rice or mustard oil.

Shanks and De (1931), in the course of post-mortem examinations, observed an extensive vascular dilatation in deep layers of the skin just where the adipose tissue began, and they considered it a characteristic feature of the disease. A similar condition was found beneath the pericardium, the lungs, and other places where the vessels were not well supported. There was no inflammatory reaction associated with this capillary dilatation.

Chopra and Bose (1933) made detailed clinical observations on cases of epidemic dropsy which lent support to the views held by Acton and Chopra (1925). They stated that the toxins elaborated by the organisms (of the *vulgatus* group) resembled histamine in having a powerful effect on the capillaries. Continuing the discussion, the authors said, 'The ætiological factors require no further emphasis beyond the fact that our recent work tends to indicate the Gram-positive bacillus as the source of toxins partly formed outside the body and ingested as chemical poisons and partly manufactured in the human gut..... A study of the pathology of the living indicates a 'cardio-capillary crisis' as being the most serious condition present..... The path of intoxication starts with the ingestion of the affected *rice*.....'

B. C. Roy *et al.* (1933) reported their clinical observations on a number of cases. They also emphasized the importance of diet in the causation of the disease. While discussing the question of prophylaxis they remarked, 'since Marwaris and Europeans are seen to escape, the logical course is to adopt the diet of those persons'. They further observed that, as a rule, 'members of an affected household who ate loaves and *chapaties* did not suffer from the disease'.

Recently, Kirwan (1935) has given support to the histamine-intoxication theory by isolating histamine from fluids of epidemic dropsy glaucoma.

Acton (1932) was so convinced about the rice theory that his last contribution on the subject dealt with the classification and grading of different varieties of Indian rice in connection with epidemic dropsy problems. Knowles (1934) summarizes the results obtained at the Calcutta School of Tropical Medicine in the following words:—'Epidemic dropsy then is due to eating diseased parboiled rice (*balam* rice) and comes about by the action of toxins absorbed from this diseased rice. (In a very few instances only it seems possible that the patient's intestine may become infected with the rice bacillus concerned, his fæces become infective, and direct person-to-person transmission occurs by the contaminative route. This, however, is very exceptional and not the rule.) In the towns decomposition generally occurs after the rice is prepared, and therefore this disease is seen during the monsoon months. In villages where the rice is stored in small store houses (*morais*) decomposition occurs when the *morai* becomes damp, the decomposed paddy is not eaten until November or December and this is the time when epidemic dropsy is seen in villages. Beri-beri on the other hand is an avitaminosis plus the action of toxins produced in rice by decomposition. In the water or glycerine test the diseased grains have an opaque, white look, like plaster of paris; healthy grains are semi-translucent and vary in translucency according to the different grades'.

A striking confirmation of the work done at the Calcutta School of Tropical Medicine is to be found in a report from Sierra Leone by Burnett (1933) who on the basis of his clinical, experimental and other laboratory studies on the 'problems of œdema in Sierra Leone' concluded that 'œdema at the Free Town Prison,.... is essentially a disease of auto-intoxication, due to absorption of highly poisonous, nitrogenous, putrefactive products, engendered in old rice, by the activity of organisms acting in hot and moist conditions and producing bodies of a nature allied to the histamine-tyramine groups. The toxic substances are water-soluble,

especially in hot water, and they are capable of withstanding boiling water, without considerable alteration of their poisonous principles. The disease-producing grains have a musty odour and generally with discoloration display the presence of decomposition and of fungoid activity. Consumption of such food-stuffs is fraught with great danger, resulting *inter alia* in the rapid onset of oedema of an extremely fatal nature'.

In spite of so much experimental and field work in support of the rice theory, some prominent practitioners still adhered to the infection theory (Editor, *Journal of Indian Medical Association*, 1934). The chief reasons for this belief were :—

- (1) Seasonal incidence in July associated with continuous rains and humidity,
- (2) widespread epidemicity,
- (3) greater incidence amongst people on riverside, suggesting water-borne infection,
- (4) occasional incidence of disease amongst persons who come in contact with epidemic dropsy, environmental and dietetic factors being excluded,
- (5) frequent spread from town to village, and
- (6) recurrent and chronic course of the disease.

Towards the end of 1934, a very severe outbreak of dropsy was reported from the Manbhum District, Bihar. Once again the medical profession showed a keen interest in this hitherto unsolved and mysterious disease. Discussions on epidemic dropsy were held at the Calcutta School of Tropical Medicine, under the auspices of the Calcutta Branch of the British Medical Association (1935). Some members stressed the infective origin of the disease, others thought that it was a histamine intoxication, probably through diseased rice. In winding up the discussion the President remarked that 'up till now we have conceived many theories but have not succeeded in bringing to light anything new. Lieut.-Colonel Kirwan's isolation of histamine from the glaucoma fluids in these cases is a distinct advance on the subject'.

The *Indian Medical Gazette* of September 1935 records some very interesting observations from different workers on the subject. Chopra and Choudhuri (1935) refer to an outbreak at the camp of the engineering students at Purulia. They state that '83 per cent of the Hindu students in the camp were affected while all the Muslims escaped. The disease could not be contagious nor water-borne, as the students of both the classes mixed freely with one another and had the same water supply. The diet was the principal factor of difference between the two classes, provisions being supplied by different contractors. The rice used by the affected students was heavily infected. The outbreak was explosive in character.....There was no spread of the disease to the students' families nor in the hostel of the Bengal Engineering College to which they returned after the epidemic'. Unfortunately the condition of the rice consumed by the unaffected group was not stated.

They carried further experimental work and stated that the emulsion of sarcoids injected into animals and human volunteers gave negative results.

Feeding experiments were also performed ; thus Chopra (1935) records that 'one monkey was fed for a period of two months with negative results on vegetables cooked in suspected mustard oil obtained from an affected family. Another monkey received injections of 5 c.c. of blood obtained from epidemic dropsy patients twice a week for two months with negative results. A human volunteer was fed on infected rice for 10 days and subsequently on food cooked with mustard oil for the same period, but did not develop the symptom of the disease. Several families in Calcutta who came in intimate contact with epidemic dropsy cases were carefully watched. There was no spread of disease among them'.

A. T. Roy (1936) gives a striking example of a semi-isolated community in which rice as a causal agent could be excluded. The Leper Colony at Purulia to which he refers has 800 inmates. They live in six separate groups. The rice supply is common to all, but two of these groups (healthy boys and healthy girls) obtain their mustard oil and vegetables, etc., from a dealer who supplies oil extracted in *kulus* (indigenous oil-pressing machine). The other sections are supplied with mill-oil ; 70 per cent of the latter went down with epidemic dropsy, while the members of the first two groups entirely escaped.

The latest confirmation of Acton and Chopra's findings regarding the association of Gram-positive, spore-forming, proteolytic bacilli with cases of epidemic dropsy and their presence in the 'opaque' or 'diseased' rice grains is to be found in a recent communication by Pasricha *et al.* (1936). Their studies included patients of epidemic dropsy and healthy individuals. They found characteristic distribution of positive stools and of positively reacting sera in their two groups which demonstrated a definite relationship between the organisms and the disease. Referring to their serological findings the authors conclude that they 'are an additional link in the chain of evidence to show that epidemic dropsy is of an infective nature and is associated with the presence of certain organisms in the rice grains. It is possible, however, that the rôle played by these organisms in the ætiology of the disease is of the same nature as that of the proteus group of organisms in typhus fever'.*

On the other hand, in a recent paper, J. K. Dutt (1936), as a result of his field investigations in the district of Backergunj, followed by a few animal experiments, concludes that *taramira* mustard oil is responsible for producing epidemic dropsy.

This brief summary of the epidemiological literature on the subject will serve to show that on account of the sickness and disablement caused by it on a large scale, epidemic dropsy is one of the major problems in the three eastern provinces of India, and particularly so in Bengal. It further shows that, even though the rice theory is at present widely accepted and forms the basis of administrative action by the Public Health Authorities and guides many practitioners in their professional care of the patients, there is an influential body of physicians who are either sceptical or are strongly opposed to it ; many, in fact, declare their adherence to the various rival theories mentioned above. It would perhaps be more correct to say that none of those who have studied the subject in some detail, feel fully satisfied with any one theory as affording a

* As a result of later studies they doubt the correctness of the interpretations which they had put on their serological observations.

complete explanation of the observed facts. We agree with the Editor of the *Indian Medical Gazette* (1935) when he says that 'many theories regarding the cause of the disease have been formulated, have lived their day, and become history and have been revived again'. Thus there is still a mystery surrounding this disease which makes it a fascinating subject for study.

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176 *Investigations into the Epidemiology of Epidemic Dropsy.*

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part II.

A SUMMARY OF FIELD STUDIES.

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In this paper, we propose to present some of the data of general epidemiological interest which have been collected by us according to a set scheme, in the course of field investigations in seven different centres.

METHOD OF COLLECTION AND ANALYSIS OF DATA.

Two schedules for recording information, one for individuals and the other for families, were prepared. They were especially designed for facilitating mechanical analysis by means of Powers Samas punching and sorting machines (see Appendix I). The investigators made house-to-house visits. They interrogated, so far as possible, each individual and recorded the information so obtained direct in the schedules according to standard instructions, thus eliminating errors arising out of copying or depending on memory. The schedules were edited immediately afterwards to ensure uniformity, completeness, and removal of obvious mistakes. A certain percentage of the schedules filled by assistants were verified by the junior author (S. C. R.) by revisiting the subjects and going over the whole

schedule with them. These checks were necessary not only to prevent possible vitiation of the data through negligence or inefficiency of workers, but also to remove any chances of errors arising out of personal bias of individual investigators.

At three localities [Gourangdih, Patherkandi, and certain parts of Calcutta (Calcutta 'A')] information was collected for every individual and for each family whether stricken with epidemic dropsy or not.

At Karimganj, at Sylhet, at another area in Calcutta (Calcutta 'B'), and at Twenty-Lines Tea Garden of the Hathikhira Tea Estate the investigations had to be restricted to the affected families and such unaffected families as had been in contact with cases of epidemic dropsy or had shared certain articles of food with the stricken families. At Jamshedpur the investigation was limited mainly to the sources of supply of mustard oil and rice to the affected families.

Field staff.—This consisted of the junior author and two junior medical men, usually assisted by a laboratory assistant.

The centres.—Short notes on the physiography, population, communications, trade, climate, and history of the outbreak for each centre are given below. Detailed meteorological data obtained from observatories situated nearest to the centre or in more or less similar regions are set out in Appendix II.

1. GOURANGDIH (period of investigation: 11th February, 1935, to 27th March, 1935). It is a medium-sized village in the Manbhum District of Bihar. It lies in a dry undulating country between two hill streams, the Darakeswari and the Dangra, which remain dry for the greater part of the year. It can be easily approached by a metalled road from Bankura and Purulia or by rail, being only at a distance of one mile from Indrabil station on the B. N. Railway.

KASHIPORE, an important market centre, is only at a distance of seven miles from this place and the two are connected by a metalled road. The population which numbers 1,054 is of Bengalee extraction and social habits. Agriculture and petty trade are the chief occupations. The climate is hot and dry for the greater part of the year.

No case of epidemic dropsy is said to have occurred in the village within living memory previous to the present outbreak. The first case was a married girl who returned from Kashipore to her mother in the village towards the end of October 1934. The whole of her father-in-law's family (at Kashipore) including herself was at the time suffering from epidemic dropsy. The disease was, in fact, widely prevalent throughout the Sadar subdivision of this district and seemed to be spreading from Purulia to the distant villages. None of the three members in the mother's family contracted the disease after the arrival of the girl. The village in fact remained free from epidemic dropsy for three weeks when another case was imported into a grocer's family from a village called Jaipur. This girl was one of the four members of the family party who had visited Jaipur and had lived with an affected family for about three weeks. Some members of the family had stayed behind at Gourangdih but after the return of the visitors had naturally to come in intimate contact with the patient and her companions. Within about two weeks cases began to develop both amongst those who had gone to Jaipur and those who had not done so. Further cases developed amongst the neighbours belonging to the same community in the southern part of the village, the first two

families to be affected being those of grocers. A fourth family of grocers living at the northern end of the village was the next to be afflicted. The grocer families of this village besides having social intercourse amongst themselves were in frequent communication with Kashipore on account of both business and social ties. The next sufferers were two Mohammedans who were particular friends of one of the grocers who had been attacked. They had spent a great deal of time with him though they lived in a different quarter of the village along with other Mohammedans. Soon after, the disease spread to another quarter where the 'karmakars' (iron smiths) lived. The first man to be attacked amongst this community was also a close associate of the grocer and the two Mohammedans referred to above. The rest of the Mohammedans, who formed a compact group of twelve families and who had remained free from the disease, suddenly became afflicted during the latter part of the *Ramzan* (the fasting month), and all the twelve families of this community were involved. In course of time, out of 193 families in the village 72 were afflicted. The epidemic came to an end on the close of February of next year. The most striking feature of the epidemic was the fact that the disease was confined to the better classes, not a single case having occurred amongst the 400 'bauries', 'bagdis', 'muchis', fishermen, or other lower classes, although their houses were interspersed between those of the victims.

2. **PATHERKANDI** (period of investigation : 19th May, 1935, to 2nd July, 1935). This is the headquarters of a small revenue circle in the Longai valley, an important tea-growing district in south Sylhet (Assam). It commands a certain amount of trade amongst the labourers of the surrounding tea gardens and as such forms a small market place. It is about 18 miles south of Karimganj which is an important business centre and is connected with it both by rail and by road. The village can be divided into two more or less distinct parts, the southern portion being residential and the northern, the market area. The total population is 476. The bulk of the people come from the surrounding villages and are Bengalee in extraction and social outlook. There are, however, a few Marwaris and up-country men besides the Monipuries who are the only indigenous population of the village. The latter form a small colony at some distance from the village proper. The residential area is occupied mostly by Government employees. The Longai river winds along the western boundary of the village, the country is plane, the drainage is not very satisfactory, and the sub-soil water-level is high.

Patherkandi had remained free from epidemic dropsy till the first week of April 1935. Here also the first case was a grocer who lived in the market area and who gave a definite history of intimate contact with epidemic dropsy patients at Karimganj. Some of the earlier cases seem to have developed amongst the close associates of the grocer, though all of them cannot be traced to him, nor the chain of cases can be shown to be complete. The residential area was soon invaded, but the first case there was a boy servant who was an intimate associate of the affected grocer. Subsequent cases in this area appeared to be confined to a small group of well-to-do people who had intimate and frequent social intercourse, though they belonged to different communities.

3. **CALCUTTA** (period of investigation : 20th August, 1935, to 30th September, 1935). During the middle of the year 1935, the southern part of Calcutta

experienced rather a severe epidemic of dropsy. Two localities in the affected area were selected. In both of them there was a fair proportion of non-Bengalee residents living side by side with the Bengalees. One of these localities consisted mostly of 'bustee' and poor class houses. The other was inhabited by better class people. The investigations were especially directed towards the detection of cases, if any, amongst the non-consumers of rice or mustard oil, or both. Another object of this inquiry was to ascertain the incidence of the disease amongst Bengalees and non-Bengalees living in the same neighbourhood. But for one Bengalee patient who stated that for the last ten years he had not taken rice more than once a week on an average we did not come across cases amongst non-consumers of rice or mustard oil. All the twenty-four victims of epidemic dropsy that were found amongst the non-Bengalees, out of a total of 977 such persons examined, were like the Bengalees in their dietetic habits.

4. KARIMGANJ (period of investigation : 8th October, 1935, to 29th November, 1935). This is a small town with a population of 5,356. It owes its importance to its being the headquarters of a subdivision of Sylhet district, and a market place. It is a railway junction on the Assam Bengal Railway. The Kushiara, a navigable river, divides the town into the market area on the east and the residential area on the west. Being an important commercial centre, it is in frequent communication with the surrounding districts and with distant villages by road, by rail, and by river. Rainfall is very heavy, amounting to 174·75 inches per year on an average. The drainage is good, but the sub-soil water-level is high and the houses are built on high plinth, the earth being obtained by excavating a tank in the compound. Conservancy system is in vogue and works fairly efficiently. A tank in every compound is a special feature of this town and this forms the main source of water supply for the household, but for drinking purposes water is obtained mostly from reserved tanks or wells. The population consists mostly of people of Bengalee extraction and habits, but the Marwaris and other up-country men form an important community, especially in the market area.

From August 1934, epidemic dropsy seems to have become endemic at Karimganj. During this month, 23 cases were reported. For a time no further cases occurred, but in November a fresh crop of cases appeared involving six families. Again in August next year, the disease broke out in an epidemic form. The present investigations relate to this outbreak. A wholesale grocer was the first victim of a series of 92 cases which developed in the course of two months. In their spatial distribution, the affected families were widely separated and no connecting link between the affected families could be established. We came across three instances in which a house was divided into separate flats, these flats being occupied by different families which had descended from a common stock. Some of these families had multiple cases, others completely escaped. In another instance, members of a joint family of grocers lived in three different groups in three localities, viz. :—

- (1) In the market area.
- (2) In the residential area.
- (3) In the village home, about three miles away from the town.

The first and the third groups were affected more or less simultaneously whereas the second entirely escaped, though there was very frequent communication among these groups. At that time there was a number of cases in both parts of Karimganj but in the village no other family was affected.

The epidemic reached its climax within a month of its onset and subsided before the end of the second month. None of the cases gave a previous history of the disease.

5. **SYLHET** (period of investigation : 1st December, 1935, to 17th January, 1936). It is the chief town in the Surma valley. It is spread over more than five square miles on a number of small hillocks separated by low valleys. Rail, road, and the Surma river provide good means of communication locally and with distant parts. The population is similar to that of Karimganj. Municipal water supply is laid. Rainfall is, on an average, 162 inches in the year. The drainage is fairly satisfactory. Excreta are removed by the conservancy system and disposed off by trenching.

There was an outbreak of epidemic dropsy at Sylhet about 30 years ago. This was, however, confined to the jail. The town had had no experience of the disease till the present epidemic in September 1935. A Mohammedan young man was the first victim. He denied having had any contact with a case of epidemic dropsy. However, within a fortnight four of the 12 members of his family were stricken. So far the cases were confined to this family but in the course of the next three weeks two other persons were attacked in different parts of the town, neither of whom gave any history of contact with a previous case. Stray cases then appeared in different parts of the town and by the beginning of November the epidemic had fully developed. It continued for more than a month after which it began to decline. The last case had been recorded before the end of the year.

6. **TWENTY-LINES TEA GARDEN** (period of investigation : 22nd April, 1936, to 29th April, 1936). This is an isolated community composed of tea-garden labourers, one European manager, three Bengalee families, and a family of grocers hailing from Bihar. They live on a small hillock in Longai valley, 18 miles to the south of Kalkalighat, a small terminal station on a branch line of the Assam Bengal Railway. The labourers are recruited mostly from distant provinces, such as the United Provinces, Bihar and Orissa, and some from the Central Provinces, and have no local associations. The only contact they have with the outside world is at the small Sunday market which assembles near the Lines, from where they obtain their meagre supplies. On occasions, perhaps not amounting to more than once a month for a family, the coolies might go to Hathikhira, a distance of two miles from the Lines, to satisfy their special requirements. Here a much bigger market assembles on Sundays.

The epidemic under report was their first experience. The investigation was commenced a fortnight after the occurrence of the first authentic case but it was not possible to definitely make out if there was a case previous to this. A coolie woman claimed to be the first to develop the symptoms about the end of March this year; she was, however, never too ill to stay away from her work. Her symptoms were indefinite and no one was able to verify her statement.

Possibly she was the first case, but the first authentic case was a Bengalee Hindu clerk who developed symptoms on the 7th of April. Almost contemporaneously with this, the female members of the Bengalee Mohammedan clerk who lived next door to him complained of typical symptoms of epidemic dropsy. This attracted the attention of the Medical Officer of the Garden, who on investigation found some stray cases amongst the labourers. Altogether 18 definite and five doubtful cases were recorded. The last case occurred on the 22nd April, 1936.

SYMPTOMATOLOGY.

The following is a brief statement of the symptoms observed in the course of our investigations:—

- (1) Usually the first symptom was either a rise of temperature or intestinal disturbance or both, the intestinal disturbance being the more frequent. In some cases swelling of feet was the first symptom observed.
- (2) Œdema of feet and legs, pitting on pressure, was present in every case for some time in the course of this disease. The œdema was most marked in the evening.
- (3) Dyspnœa with exertion, and in advanced cases without exertion, and a corresponding degree of cardiac involvement was frequently seen.
- (4) Patchy pigmentation of the face was especially marked at Gourangdih.
- (5) Telangiectatic growths, bleeding on slightest manipulation, were observed in a few cases.
- (6) Occasional bleeding from mucous surfaces, especially from the rectum was noticed.
- (7) Condition of the knee-jerks: These were exaggerated in some, absent in a few but more or less normal in the majority of cases.

N.B.—In contrast to the symptoms which were observed in all the epidemics included in our discussion, there was a small outbreak in an Assam tea garden involving a dozen or more individuals in which the main symptoms were referable to the nervous system, such as intense tenderness of the calf muscles, inability to stand up or walk on account of pain, and loss of knee-jerk. The cardiac involvement was also marked.

Analysis of data.

With a view to present our data in an easily comprehensible form we propose to describe them as distributions classified according to different criteria. The data from various investigations have been combined or presented separately according as one or the other type of treatment appeared more appropriate. As a rule, combined data are set forth where the investigations included both the affected and the unaffected families. Where the study was confined to the affected families or such other families as were in some way connected with them, the data regarding the affected families have been treated together with the affected families of places where all the families were investigated.

Distribution according to religious communities (vide Table I).

TABLE I.

Distribution according to religious communities.

Area.	HINDU.		MOHAMMEDAN.		CHRISTIAN.		TOTALS.	
	Number of persons.	Percentage attacked.	Number of persons.	Percentage attacked.	Number of persons.	Percentage attacked.	Number of persons.	Percentage attacked.
Gourangdih ..	965	13.7	89	51.7	0	..	1,054	16.9
Patherkandi ..	302	10.9	175	10.9	0	..	477	10.9
Calcutta ..	1,921	9.6	54	0.0	73	0.0	2,048	9.0
Karimganj ..	4,022	2.2	1,298	0.0	36	13.9	5,356	1.8
Sylhet ..	11,453*	0.9	9,982*	1.0	21,435	0.9
Twenty-Lines ..	733	2.3	10	60.0	1	0.0	744	3.1

* These represent the 1931 census figures; all non-Mohammedans are included under the heading Hindus. In other places the data were obtained by house-to-house visits.

Two main points emerge from a perusal of this table, viz.:—

- (1) That the incidence in both the two major communities varied from place to place.
- (2) That in some places (Gourangdih and to some extent at Sylhet) the Mohammedans suffered more than the Hindus, while in others the majority of the victims were Hindus. The example of Karimganj is particularly striking. Here the Mohammedans formed one-fourth of the total population, but not one of them was stricken. The peculiarities in the mode of living of the two communities do not, therefore, appear to be associated with the incidence of the disease. The variation is probably dependent upon the aggregation or otherwise of the houses of each community. Thus, when the Mohammedans formed a compact group as at Gourangdih and Karimganj, they were either badly afflicted or completely escaped, while at Patherkandi and Sylhet, where the Hindu and Mohammedan houses were intermixed, the incidence in the two communities was more or less the same.

TABLE II.

Distribution of Hindus (excluding non-Bengalees) according to caste, shewing cases and the total population.
(a) Gourangdih.

	Brahmin.	Baidya.	Kayastha.	Bania.	Karnahkar.	Sarak.	Kulu.	Fishermen.	Bagdi and Bauri.	Muchhi.	Others.	TOTALS.
Cases ..	11	15	2	25	55	5	13	0	0	0	6	132
Total populations ..	24	67	5	136	148	61	34	33	293	74	90	965
Percentage affected ..	45.83	22.39	40.0	18.38	37.16	8.197	38.24	0.0	0.0	0.0	6.67	13.68
Expected number ..	3.28	9.16	0.68	18.60	20.24	8.34	4.65	4.51	40.08	10.12	12.31	..
Ratio observed expected ..	3.35	1.64	2.94	1.34	2.72	0.60	2.80	0.0	0.0	0.0	0.49	..

TABLE II—*contd.*(b) *Patherkandi.*

	Brahmin.	Baidya.	Kayastha.	Vaishya and Bania.	Gowala and Namasudra.	Muchi.	Others.	TOTALS.
Cases ..	2	9	12	10	0	0	0	33
Total persons ..	31	28	99	63	9	25	33	288
Percentage affected ..	6.45	32.14	12.12	15.87	0.0	0.0	0.0	11.46
Expected number ..	3.55	3.21	11.34	7.22	1.03	2.87	3.78	..
Ratio $\frac{\text{observed}}{\text{expected}}$..	0.56	2.80	1.06	1.39	0.0	0.0	0.0	..

(c) *Calcutta.*

	Brahmin.	Baidya.	Kayastha.	Vaishya and Banikya.	Teli, Carpenter, Barber, and Blacksmith.	Jogi and Kambojen.	Kahar, Muchi, Rupdas, and Dusad.	Tambul and Gowala.	Others.	TOTALS.
Cases ..	50	2	41	50	0	4	0	13	1	161
Total persons ..	216	22	234	365	45	18	71	29	54	1,054
Percentage affected ..	23.15	9.09	17.52	13.698	0.0	22.22	0.0	44.83	1.85	15.28
Expected number ..	32.99	3.36	35.74	55.75	0.87	2.75	10.85	4.43	8.25	..
Ratio $\frac{\text{observed}}{\text{expected}}$..	1.52	0.555	1.15	0.897	0.0	1.45	0.0	2.93	0.12	..

TABLE II—*concd.*
(d) *Karinganji.*

	Brahmin.	Baidya.	Kayastha.	Vaishya Banikya.	Sweeper, Patney, and Malakar.	Muchi and Gowala.	Namasudra.	Others.	TOTALS.
Cases ..	34	2	30	21	0	0	2	0	89
Total persons ..	634	217	1,419	1,278	227	118	42	32	3,967
Percentage affected ..	5.36	0.92	2.11	1.64	0.0	0.0	4.76	0.0	2.24
Expected number ..	14.22	4.87	31.84	28.67	5.09	2.65	0.94	0.72	..
Ratio $\frac{\text{observed}}{\text{expected}}$..	2.39	0.41	0.94	0.73	0.0	0.0	2.13	0.0	..

(e) *Sylhet (the survey area).*

	Brahmin.	Baidya.	Kayastha.	Vaishya.	Sweeper, Patney, and Malakar.	Muchi.	Gowala.	Others.	TOTALS.
Cases ..	3	0	3	5	0	0	0	1	12
Total persons ..	183	143	538	343	85	29	41	59	1,421
Percentage affected ..	1.64	0.0	0.56	1.46	0.0	0.0	0.0	1.69	0.84
Expected number ..	1.55	1.21	4.54	2.897	0.72	0.24	0.35	0.498	..
Ratio $\frac{\text{observed}}{\text{expected}}$..	1.94	0.0	0.66	1.73	0.0	0.0	0.0	2.01	..

Distribution amongst the Hindus (excluding non-Bengalees) according to caste
[vide Tables II(a), II(b), II(c), II(d), and II(e)].

The caste distribution is characteristic. In the villages the disease is confined to the higher castes, viz., Brahmins, Baidyas, Kayasthas, and Banias, which are generally economically well off, while in the towns some of the lower castes are equally affected. This suggests that the distribution of the disease according to caste is due to the linkage of this factor with economic status.

Distribution according to the province of origin.

The relevant information is given in Table III where the expected number in each case has also been shown for purposes of comparison. These numbers have been calculated by multiplying the population of each provincial group with the ratio of total cases for the whole group to the total population in the area. A very high incidence amongst the Bengalees is obvious.

TABLE III.

Cases and non-cases amongst persons from different provinces of India residing in a certain area of Calcutta (combined data for 'A' and 'B').

	Bengal.	Assam.	Bihar.	Orissa.	U. P.	Punjab.	Madras.	Bombay.	Other non-Bengalee Indians.	TOTALS.
Cases ..	161	0	4	0	3	1	2	0	14*	185
Totals ..	1,071	11	389	17	88	226	107	60	79	2,048
Expected number.	96.75	0.99	35.14	1.54	7.95	20.42	9.67	5.42	7.14	..
Ratio between observed and expected.	1.66	0.0	0.11	0.0	0.38	0.048	0.21	0.0	1.96	..

* All patients belonged to one family.

A closer examination showed that all the 24 non-Bengalee patients had been resident in Calcutta for over twenty years or had been settled there for more than a generation and had taken to the mode of life and dietetic habits of the Bengalees. The only exception was that of a Punjabi who had been in this city for six or seven years only but having married a Bengalee woman had greatly changed his diet.

Distribution according to age [vide Tables IV(a) and IV(b)].

TABLE IV(a).

Distribution of cases according to age (combined data for Gourangdih, Patherkandi and Calcutta 'A').

Age groups :—	0—	$\frac{1}{2}$ —	1—	3—	5—	15—	25—	35—	45—	55—	65—	TOTALS.
Cases ..	0	0	0	1	68	66	75	41	31	13	6	301
Totals .	18	26	125	108	405	447	398	219	162	67	31	2,006
Expected number.	2.7	3.9	18.8	16.2	60.8	67.1	59.7	32.9	24.3	10.1	4.7	..
Ratio between observed and expected.	0	0	0	0.06	1.1	0.98	1.3	1.2	1.3	1.3	1.3	..

$\chi^2 = 17.46$

P = less than 0.01 (significant).

TABLE IV(b).

Distribution of cases and non-cases according to age (combined data for all the affected families of the different places of investigation).

Age groups :—	0—	$\frac{1}{2}$ —	1—	3—	5—	15—	25—	35—	45—	55—	65—	TOTALS.
Cases ..	0	0	1	6	199	190	152	90	64	27	13	742
Totals ..	14	19	80	94	406	394	281	185	118	59	42	1,692
Expected	6.1	8.3	35.1	41.2	178.0	172.8	123.2	81.1	51.7	25.9	18.4	..
Ratio between observed and expected.	0.0	0.0	0.03	0.15	1.11	1.10	1.23	1.11	1.24	1.04	0.71	..

$\chi^2 = 85.74$

P = less than 0.01 (significant).

The age constitution together with the actual incidence in each age group and the expected numbers are shown in Tables IV(a) and IV(b). In both cases there is a significant disparity between the actual and the expected distributions as shown by the two tests.

The toddlers were only exceptionally affected, young children were only mildly touched, the adolescents gave a higher incidence than expected, those in the prime of life were the worst sufferers, and the incidence again tended to drop with advance in age. This characteristic age distribution is so regular and striking that any theory regarding the ætiology of this disease which fails to account for it should not be seriously considered.

Distribution according to sexes.

There is a difference of opinion amongst various observers with regard to the relative incidence of epidemic dropsy in the two sexes. The relevant data from our series are given in Tables V(a) and V(b).

TABLE V.

Distribution of cases in the two sexes.

(a) *Combined data for Gourangdih, Patherkandi, and Calcutta 'A'.*

	Male.	Female.	TOTALS.
Cases	162	139	301
Totals	1,139	872	2,011
Case incidence per cent ..	14.2	15.9	15.0

$$\chi^2 = 1.144$$

P = less than 0.30 (not significant).

(b) *Combined data for the affected families of all the places.*

	Male.	Female.	TOTALS.
Cases	415	327	742
Totals	960	733	1,693
Case incidence per cent ..	43.23	44.61	43.83

$$\chi^2 = 0.3225$$

P = between 0.7 and 0.5 (not significant).

According to our data, therefore both the sexes are equally liable to be affected.

TABLE VI.

*Distribution of cases according to occupation.**(a) Combined data for Gourangdih, Patherkandi, and Calcutta 'A'.*

	Agent to landlord.	Cultivator.	Food handler and milkman.	Menial servant.	Medical or nursing.	Student or clerk.	Housewife.	Trader, artisan and shopkeeper.	Beggar.	'At home'.	Sweeper, cartier, and others.	Totals.
Cases ..	2	7	3	17	2	57	97	60	1	51	4	301
Totals ..	10	39	41	187	18	285	491	323	3	593	21	2,011
Expected number ..	1.497	5.84	6.14	27.99	2.69	42.66	73.49	48.35	0.45	88.77	3.14	..
Ratio between observed and expected.	1.34	1.199	0.49	0.61	0.74	1.33	1.32	1.24	2.22	0.57	1.27	..

(b) Combined data for the affected families only (Sylhet and Twenty-Lines families excluded).

	Agent to landlord.	Cultivator.	Food handler and milkman.	Menial servant.	Medical or nursing.	Student or clerk.	Housewife.	Trader, artisan and shopkeeper.	Beggar.	'At home'.	Sweeper, cartier, and others.	Totals.
Cases ..	2	7	7	26	9	131	158	89	1	73	6	509
Totals ..	3	9	20	69	17	226	278	172	2	306	8	1,110
Expected number ..	1.38	4.13	9.17	31.64	7.80	103.63	127.48	78.87	0.92	140.32	3.67	..
Ratio between observed and expected.	1.45	1.69	0.76	0.82	1.15	1.26	1.24	1.13	1.09	0.52	1.63	..

Distribution of cases according to the vocation of individuals
[vide Tables VI(a) and VI(b)].

The data for some occupations are not sufficiently large to give a correct idea of occupational distribution but it would appear that epidemic dropsy claimed most of its victims from amongst the following groups :—

- (1) Students or clerks and landlords' agents.
- (2) Housewives.
- (3) Traders or artisans.
- (4) Cultivators.

These groups form the bulk of the middle classes. On the other hand those included under the headings 'at home', food handlers, and menial servants fared well.

Distribution according to economic status (vide Table VII).

The factors that were taken into consideration to decide the economic status of the families were :—

- (i) Occupation of the head of the family,
- (ii) Clothing and nourishment of the children,
- (iii) General condition of the house.

TABLE VII.

Incidence of epidemic dropsy according to the economic status of the families :—

(a) Combined data for Patherkandi and Calcutta 'A'.

	Poor.	Middle.	Upper middle and rich.	TOTALS.
Affected	4	28	3	35
Totals	94	103	7	204
Expected number ..	16.13	17.67	1.20	..
Ratio between observed and expected.	0.25	1.58	2.5	..

(b) Affected families of Karimganj, Sylhet and Calcutta 'B'.

Poor	22
Middle	101
Upper middle	1
TOTAL	124

It is clear from this table that epidemic dropsy is chiefly the disease of middle classes. The poor people suffer relatively much less. None of the Marwari families, however, suffered.

Distribution according to dietetic habits [vide Tables VIII(a) and VIII(b)].

A broad division into vegetarians and non-vegetarians was made, only those refraining from taking meat, fish, or eggs being included in the former group.

TABLE VIII.

Cases and non-cases amongst vegetarians and non-vegetarians :—

(a) *Combined data for Gourangdih, Patherkandi, and Calcutta 'A'.*

	Vegetarians.	Non-vegetarians.	TOTALS.
Cases	24	277	301
Totals	278	1,617	1,895
Case incidence per cent ..	8.63	17.13	15.88

$$\chi^2 = 12.819$$

P = less than 0.01 (significant).

(b) *Affected families from all the places investigated.*

	Vegetarians.	Non-vegetarians.	TOTALS.
Cases	38	704	742
Totals	100	1,527	1,627.
Case incidence per cent ..	38.00	46.10	45.61

$$\chi^2 = 2.48$$

P = between 0.1 and 0.2 (not significant).

These tables are of considerable interest. They show that contrary to the popular belief vegetarians had a definite advantage over those in the habit of taking animal food. However, even strict vegetarians like Hindu widows may sometimes suffer. Further it appears [vide Table VIII(b)] that factors common to the members of the family are more important than merely the food habits of individuals.

The distributions having a direct bearing on the three important theories regarding the ætiology of the disease, viz. :—

(1) The rice theory,

(2) The mustard-oil intoxication theory, and

(3) The contact infection theory

may now be discussed.

1. *Distribution in relation to the rice theory.*

(i) There are two processes in the extraction of the grain from the paddy which are of interest to us, viz. :—

(a) Preparation of paddy for husking.

(b) The method of husking.

Paddy may either be parboiled or sun-dried before husking. Except in the case of Hindu widows who, for certain religious reasons, always take sun-dried rice, the habit of taking one or the other type of rice by the people is merely a matter of local custom. Thus, while in Bengal and Bihar the usual custom is to take parboiled rice, in Assam sun-dried rice is the one commonly consumed. It is only in exceptional cases that a few individuals depart from the local habits. On the basis of these data no fair comparison in the incidence of cases amongst persons of different rice habits can be made.

The relevant data are, however, given in Tables IX(a) and IX(b) for the information of the reader.

TABLE IX.

(a) *Affected and unaffected families according to the method of preparation of paddy for husking.*

		Sun-dried.	Parboiled.	TOTALS.
Gourangdih	Affected ..	0	72	72
	Total ..	1	192	193
Patherkandi	Affected ..	15	1	16
	Total ..	125	3	128
Calcutta 'A'	Affected ..	2	16	18
	Total ..	24	42	66
Sylhet (selected area)	Affected ..	3	3	6
	Total ..	250	59	309

(b) *Distribution of affected families according to the method of preparation of paddy for husking.*

	Sun-dried.	Parboiled.	TOTALS.
Calcutta 'B' ..	1	32	33
Karimganj ..	21	1	22
Sylhet ..	58	9	67
Twenty-Lines ..	13	0	13

With regard to the process of husking, in the course of our investigations, we met with the milled rice in bigger towns only where imported grain is consumed. In rural areas where epidemic dropsy was rife, *milled rice was almost unknown*. The process of milling, therefore, as a factor in the causation of epidemic dropsy, does not seem to be of any significance. For the sake of comparing the data, however, the distribution of cases according to the consumption of milled or hand-pounded rice in parts of Calcutta and Sylhet, where both affected and non-affected families were investigated, is given in Table IX(c).

TABLE IX.

(c) *Distribution of affected and unaffected families according as they used milled or hand-pounded rice.*

		Hand-pounded.	Milled.	TOTALS.
Calcutta (A)	{ Affected ..	4	14	18
	{ Total ..	14	52	66
Sylhet (in selected area)	{ Affected ..	4	2	6
	{ Total ..	295	14	309

(ii) *Storage*.—Storage of rice is another factor that demands consideration because according to the 'rice theory' the grain has to be stored under certain conditions of temperature and humidity before the toxins can develop.

The actual method of storage of rice or paddy varies according to the local custom. At Gourangdih it is usually stored in what are called *kuchuris*. The grain is placed on a bed of straw which forms the inner lining of the *kuchuri* and wholly encloses it. A cocoon-like structure is built round it with rope, also made out of straw. They are more or less spherical in shape with the diameter varying from 2 to 4 feet. To protect them from the damp they are placed inside the rooms on a raised platform made out of bamboos or stones.

In well-to-do families the year's requirement of the grain is gradually husked and stored, after the harvest, during the winter months. The *kuchuris* serve to store the rice and the daily requirements are drawn from them by poking a stick between the coils of the rope and tilting it up to make a small temporary hole. Extra supply of grain which may be used for payment of wages or for sale is stored as paddy in what are known as *morais*. These more or less cubical structures are much bigger than the *kuchuris*, the sides varying from 4 to 6 feet and the height from 5 to 8 feet depending upon the amount of grain stored. They are constructed more or less like the *kuchuris* but being larger the sides are reinforced with pieces of split bamboos. They are built on a wooden platform raised from the ground to prevent damp. They may be placed inside the sheds or in the open compound, in which case a thatched roof is built on them to protect them from rain and sun.

Still larger quantities of grain are stored in especially constructed mud-chambers called *hamires*, built on wooden platforms with a side-door placed near the top, which is used both for putting in more paddy and for removing it.

Whatever the method used we were satisfied that the rice or paddy was thoroughly protected from the damp during storage. The poor people who do not own land or those who do not raise sufficient crop to last throughout the year obtain paddy as wages for the work they do and husk it as required. The residents of Patherkandi, Karimganj, Sylhet, and Twenty-Lines, being non-cultivators usually buy rice in small quantities from the villagers who sell the rice and paddy. The sun-dried hand-pounded rice being the one usually consumed, the husked grain is not stored for long as this variety rapidly deteriorates when stored.

At Calcutta, it was impossible to ascertain the storage period of the rice, but it can, with reasonable certainty, be assumed that almost in every case it was stored for some months before it reached the consumer. The distribution of families in relation to the habits of storing rice is shown in Table X.

TABLE X.

Distribution of families according as they used stored or fresh rice.

(a) *All the families of Gourangdih, Patherkandi, and Calcutta 'A'.*

		Freshly husked.	Stored for more than a month.	Both freshly husked and stored.	TOTALS.
Gourangdih	{ Affected ..	39	14	19	72
	{ Total ..	101	39	53	193
Patherkandi	{ Affected ..	17	0	0	17
	{ Total ..	122	7	0	129
Calcutta 'A'	{ Affected ..	0	18	0	18
	{ Total ..	0	66	0	66
Sylhet (Survey area)	{ Affected ..	4	2	0	6
	{ Total ..	298	25	0	323
Combined	{ Affected ..	60	34	19	113
	{ Total ..	521	137	53	711

(b) *Affected families of Karimganj, Sylhet, and Twenty-Lines.*

	Freshly husked.	Stored.	TOTALS.
Karimganj ..	21	1	22
Sylhet ..	56	13	69
Twenty-Lines ..	12	1	13

It would appear from Table X(a) that at Gourangdih, Patherkandi, and Sylhet a relatively small number of families used stored rice at the time of investigation, whereas in Calcutta 'A', all the families used stored rice only. From the combined data, however, it does appear that families using stored rice were at a disadvantage but the difference is not significant. However, as shown in Table X(b), a large number of people may be stricken with epidemic dropsy even though they use only freshly husked grains.

(iii) The question whether there was any grouping round of cases to a common source of supply of rice was also investigated. The relevant data are contained in Table XI.

TABLE XI.

Distribution of families according to the source of rice supply.

(a) *Affected and unaffected families.*

	SOURCE OF RICE.				TOTALS.
	Only self-grown.	Only shop.	Only stray cultivators.	Varying sources.	
<i>Gourangdih.</i>					
Affected ..	42	6	0	24	72
Totals ..	110	18	0	65	193
Expected number ..	41.0	6.7	0	24.2	..
<i>Patherkandi.</i>					
Affected ..	0	0	17	0	17
Totals ..	5	5	117	0	127
Expected number ..	0.7	0.7	15.7

(b) *Affected families at Karimganj, Sylhet, and Twenty-Lines.*

	SOURCE OF RICE.				TOTALS.
	Only self-grown.	Only shop.	Only stray cultivators.	Varying sources.	
Karimganj ..	3	0	18	1	22
Sylhet ..	2	0	59	8	69
Twenty-Lines ..	3	0	8	2	13
Combined ..	8	0	85	11	104

These data clearly bring out the fact that cases were not confined to persons obtaining their rice supply from a common source. In fact, simultaneous infection of a large number of separate stores of rice will have to be assumed if rice was to be held responsible for conveying the infection or elaborating the toxin. In

Calcutta, however, where the families obtained their rice supply from different shops no association of any group of affected families with any particular shop could be demonstrated.

(iv) *Rice-water*.—If epidemic dropsy was due to a water-soluble toxin it is reasonable to assume that the disease would be either entirely absent or rare amongst people who habitually throw away water in which the rice is boiled and that it would be relatively common amongst those who consume the rice-water. The relevant data are set out below :—

TABLE XII.

Distribution of cases and non-cases according to the use or rejection of rice-water.

(a) *All the families of Gourangdih, Patherkandi, and Calcutta 'A'.*

	Rice-water used.	Rice-water rejected.	TOTALS.
Cases	14	283	297
Totals	488	1,405	1,893
Case incidence per cent ..	2.87	20.14	15.69

$$\chi^2 = 81.76 \quad P = \text{less than } 0.01 \text{ (significant).}$$

(b) *Cases and non-cases in the affected families of the different places of investigation.*

	Rice-water used.	Rice-water rejected.	TOTALS.
Cases	30	707	737
Totals	80	1,535	1,615
Case incidence per cent ..	37.5	46.06	45.63

$$\chi^2 = 2.25 \quad P = \text{between } 0.2 \text{ and } 0.1 \text{ (not significant).}$$

The result of this analysis is contrary to what would be expected on the basis of the rice theory. In Table XII(a) incidence of cases amongst the consumers of rice-water is significantly less than in the other group. Data given in Table XII(b) are of great interest. While the throwing away of rice-water does not afford any safeguard against the occurrence of the disease factors common to the members of the family are, as also shown before (Table VIII), of greater importance than the habit regarding the use of rice-water.

N.B.—Persons who sometimes used and sometimes rejected the rice-water are excluded from the above table.

(v) 'Diseased' rice according to the 'rice theory' develops an opacity which is employed as a rough and ready test of infection of the grain. The position of the opacity varies in different samples of rice. It is claimed that the distinctive type of opacity which signifies infection is in the centre of the grain with a clear zone around. At times one meets with a linear opacity along the margin of the grain which may extend into its substance to a varying extent sometimes involving greater part of the grain. It is difficult to assume that this type of opacity is essentially different from the typically central opacity and that it is of no significance. We have, therefore, included both types of opacities in estimating the intensity of 'infection' in the samples of rice examined by us (*vide* Table XIII).

TABLE XIII.

Cases according to the percentage of opaque grains consumed at the time of investigation (data referring to Gourangdih and Patherkandi).

Per cent:—	10	20	30	40	50	60	70	80	90	100	TOTALS.
Cases ..	4	8	20	51	34	53	26	24	8	0	228
Total ..	15	51	201	290	232	283	200	105	28	0	1,405
Case incidence per cent ..	26.7	15.7	9.95	17.6	14.7	18.7	13.0	22.9	28.6	..	16.2

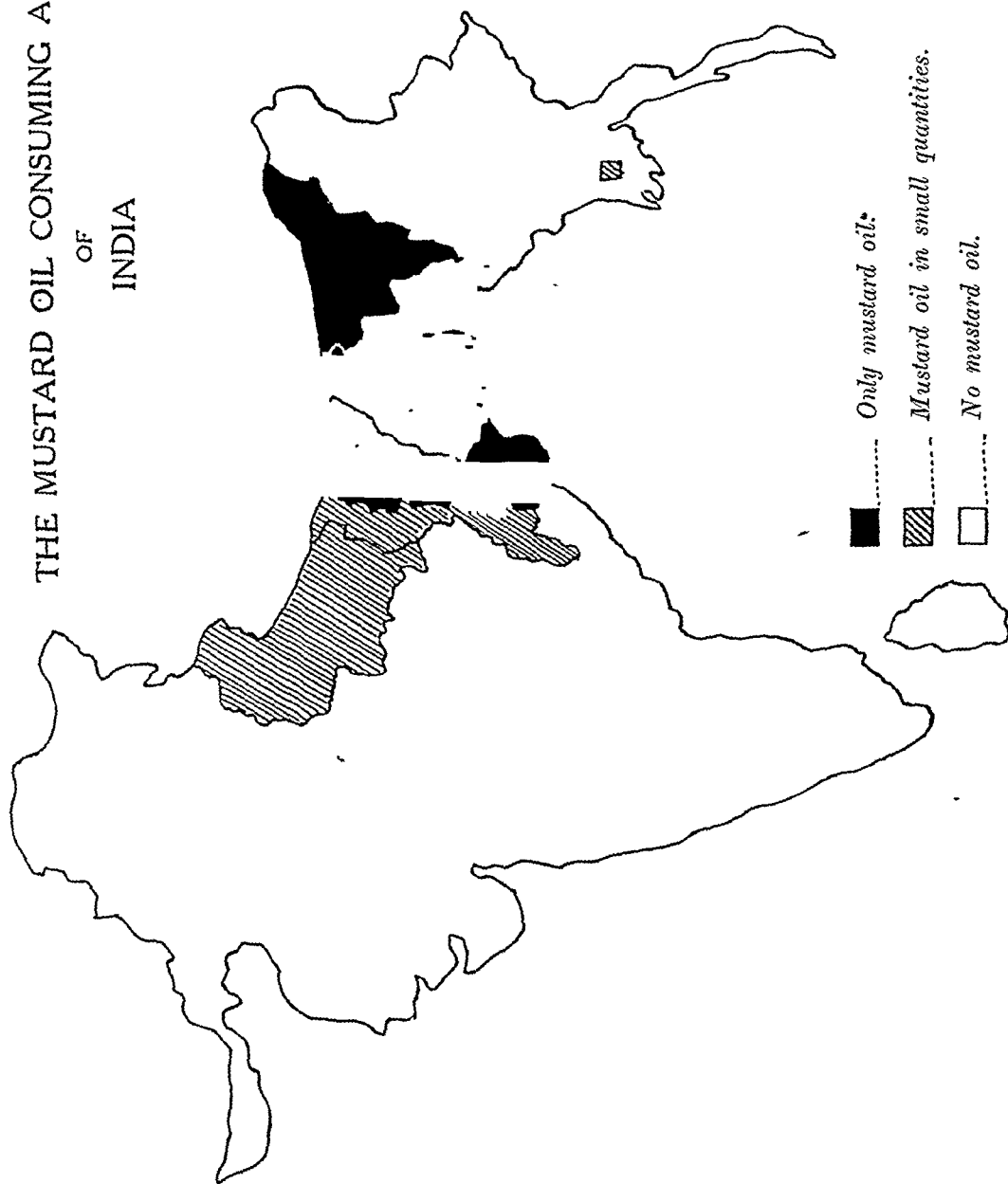
These data are not easy to interpret because the rice examined did not necessarily come from the same stock as was consumed by the victims prior to the development of cases in the family. However there is slight advantage in favour of the families not using 'heavily infected' grains but this difference is statistically insignificant, as can be shown by comparing the variation around the mean (5,884.9) and that around a straight line fitted by the least square method with the percentage of opaque grains as an independent variable (5,545.5).

At Karimganj, Sylhet, and Twenty-Lines, this opacity, especially the marginal one, was very commonly met with both in the affected and unaffected families. We have reasons to believe that the opacity of the grain is characteristic of certain varieties of rice independent of infection. We shall discuss this subject in greater detail in a subsequent communication.

2. *Analysis of data with reference to the mustard-oil intoxication theory.*

While the geographical distribution of rice eaters is pretty wide, that of the habitual consumers of mustard oil is much more restricted and the latter does, in fact, closely coincide with the epidemic dropsy area as roughly depicted in Maps 1 and 2. Similar remarks apply to age and race distribution of cases in an epidemic area. Unlike the rice theory, however, this theory lacks definition and the exact nature of the poisonous base has never been indicated. For the present purpose, it was assumed that a deleterious substance was introduced into the system through the vehicle of mustard oil. A summary of the habits of the people with regard to the use of mustard oil is given in Tables XIV(a), XIV(b), and XIV(c).

THE MUSTARD OIL CONSUMING AREAS
OF
INDIA



These tables bring out an important point, namely, that no case was seen amongst the non-consumers of mustard oil. The interpretation of these data is, however, complicated by a number of associated factors. In the first place, the distribution of the oil from the manufacturer to the consumer is complicated by the fact that the oil undergoes repeated admixtures with different brands of mustard oil or sometimes with other oils and that in this process the name of the brand also undergoes many changes. Besides, not only does the consumer, usually get his oil from more than one grocer but the grocer himself invariably secures his supply from more than one merchant or occasionally from a number of mills direct. This complicates the issue further and it is extremely difficult, either for the grocer or for the consumer, to say as to what particular brand of oil was used. In many cases the managers of the stores are unwilling to give correct information for fear of incurring odium. These difficulties are obviated when the oil is purchased by the family in the original containers. This practice is rather uncommon, but at Karimganj and at Jamshedpur instances came to our notice where the source of mustard oil could be definitely traced. In both the places, the available evidence gave strong reasons to associate a particular consignment of the oil from a known source with epidemic dropsy cases. The data relating to Karimganj are described in detail in Part V of this series (this issue). At Jamshedpur, more definite evidence associating a particular brand of mustard oil with cases of epidemic dropsy was obtained.

Jamshedpur epidemic.—Jamshedpur has a population of about 100,000 composed of people from all provinces of India and of a small number of foreigners. Altogether 222 cases involving 52 Bengalee and 14 non-Bengalee families occurred within five weeks commencing from the first week of October. The Bengalee families, as usual, were habitual consumers of rice and mustard oil, while of the non-Bengalee families, all but one had taken to the food habits of the Bengalees. Even in the remaining one family mustard oil and rice did not form inconsiderable constituents of the daily fare.

Jamshedpur receives its mustard oil supply mainly from four mills, one of which is located in the town itself. This mill had been in existence for a good many years, and had a good reputation and a large clientele in the town. Two grades of oil were supplied by this mill—the 'red' and the 'yellow' brand—the former being the superior of the two and guaranteed pure. Table XV, which sets out information regarding the use of the 'red' brand oil by the affected families, is of considerable interest.

Further, inquiries made at seven other shops which had no affected families on their clientele showed that none of them stocked the suspected oil.

These facts would show that the use of 'red' brand mustard oil obtained from the above-mentioned mill was a factor common to practically all the affected families. The association of the suspected oil with the incidence of the disease is further strengthened by the fact that all the affected families which purchased the oil in original containers did so within the period of five weeks from the beginning of October 1936 to about the middle of November 1936. Moreover, the first cases in these families occurred within one to two weeks of the commencement of the use of the suspected oil. There is one exception in which this period was about a month. These facts would appear to fit in very well with the hypothesis that

for some reason or the other a particular consignment of a well-known brand of oil may in some way cause an outbreak of epidemic dropsy amongst its consumers. It must, however, be noted that definite information was available about ten families in which the suspected brand of oil was consumed but which had remained unaffected. The dates on which the suspected brand of oil was purchased and used were not available.

TABLE XV.

Distribution of the affected families according to their source of mustard oil supply.

		From the mill direct in original containers.	From the mill direct in small quantities.	The suspected brand of oil through retail grocers.	Brand used could not be ascertained though the supplying grocer stocked the suspected oil.	The evidence regard- ing the stocking of the suspected oil by the grocer is con- flicting.	TOTALS.
Bengalees	..	11	4	24	10	3	52
Non-Bengalees	..	3	0	11	0	0	14

Another difficulty that arises in regard to the ætiological rôle of mustard oil in epidemic dropsy is the want of suitable controls due to the universality of its use as an article of diet in parts of India which are subject to outbreaks of epidemic dropsy. However, in large towns like Calcutta, Benares, Jamshedpur, etc., it has been observed that the disease is confined to the Bengalee families or to such other families as have adopted Bengalee habits of food. This point is best illustrated by Table XVI.

This table also shows that both in the Bengalee and the non-Bengalee communities increase in the amount of mustard oil consumed roughly bears a relation to the incidence of the disease. However, for equal consumption of the oil, there is a difference in the morbidity rates in the two communities. Is it due to a special vulnerability of the Bengalees to this disease which may be racial or due to rice or other food habits?

3. *Distribution in relation to the contact infection theory.*

Like the mustard-oil theory, this theory also lacks definition. The causal agent has not been demonstrated, the portals of discharge of infection and those of entry and the means of transmission have not even been suggested. The simplest

TABLE XVI.

Cases and non-cases according to the quantity (in ounces) of mustard oil consumed per head per week (Calcutta figures).

Quantity (oz.) :—	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Totals
Bengalces.	Cases ..	0	0	0	2	15	31	24	32	25	6	5	2	0	0	0	0	161
	Totals ..	13	6	80	98	93	183	122	116	112	72	10	5	14	5	11	1	971
	Case in- evidence per cent.	0.0	0.0	0.0	2.04	16.13	16.94	19.67	27.59	22.32	8.33	50.0	40.0	0.0	0.0	33.33	0.0	16.58
	Expected number.	2.16	0.99	13.26	16.25	15.42	30.34	20.23	19.23	18.57	11.94	1.66	0.83	2.32	0.83	4.97	0.17	..
Non-Bengalces.	Cases ..	0	0	0	0	0	3	4	0	2	0	0	15	0	0	0	0	24
	Totals ..	318	24	44	121	107	72	70	0	28	0	0	15	2	0	0	0	801
	Case in- evidence per cent.	0.0	0.0	0.0	0.0	0.0	4.17	5.71	..	7.14	100.0	0.0	3.00
	Expected number.	9.53	0.72	1.31	3.63	3.21	2.16	2.09	..	0.84	0.45	0.06

way in which infection may spread is by contact, for, as Chopin says 'if it can explain epidemiological phenomenon. there is no occasion for assuming the growth of pathogenic germs outside the body or of infection by air or any similar theory and no such theory should be adopted unless pretty strong evidence can be brought to its support'. The following tables give information relevant to the contact infection theory:—

TABLE XVII.

Distribution of cases according to the degree of contact.

		Social or professional contact.	Contact due to living with patient.	No known contact.	TOTALS.
<i>(a) Data referring to all the families at Gourangdih, Patherkandi, and Calcutta (A).</i>					
Cases	74	188	39	301
Totals	707	529	714	2,010
Case incidence per cent		9.65	35.54	5.46	14.98
<i>(b) Data referring to affected families only.</i>					
Cases	129	494	119	742
Totals	1,409	..	1,409

Table XVII(a) seems to indicate that contact with cases increased the liability to get the disease and that this liability increased with the intimacy of contact. But this finding is complicated by the fact that intimate family contact went hand in hand with the consumption of a common diet.

Table XVII(b), however, shows that quite a large number of cases denied any history of contact. The 119 cases must be the first cases in the affected families, for once a case develops in the family, the other cases that follow and other unaffected members of the family are to be reckoned as contacts.

In contrast to the epidemic at Gourangdih, where the sequence of events suggested imported cases as sources of infection, there were instances of importation of cases into unaffected families, two at Patherkandi, eight at Karimganj, and three at Sylhet in none of which secondary cases developed.

To test the contact infection hypothesis further it is necessary to study the data more closely. If infection was spread by contact, the state of congestion in which the family lived should bear a direct relation to incidence or, to be more precise, to the development of secondary cases in the family. The size of the family should be another determining factor. To correlate the congestion and incidence rate, it is necessary to eliminate the complication introduced by differences in size of the families. This has been accomplished by applying the method of partial correlations.

Before treating the data statistically it is necessary to eliminate certain other sources of error arising out of heterogeneity. The most important of these factors is that of age. It has been shown that children under five years are seldom affected but beyond this age all persons are more or less equally liable. Again, economic status has a bearing on the rates of incidence. The disease is practically limited to the middle class people—the poor people being almost entirely free from the attack. In working out the correlations, therefore, children under five years were excluded. As regards the economic factor, comparisons were, where necessary, restricted to families of similar economic status.

Congestion.—This was calculated on the basis of 'room per person'. Thus if α persons in one family occupied C α rooms and β persons in another family occupied C β rooms, then the congestion in both cases was C . In working this out the total number of persons living under similar states of congestion was first found out and from the cases developing (excluding the first cases in each family because here we are concerned with the spread of the disease in affected families) in those specified populations the rates of incidence corresponding to varying congestions were calculated. The table below gives the rates of incidence for different degrees of congestion.

TABLE XVIII.

Distribution of congestion and the rate of incidence in the affected families.

Congestion.	Incidence.	Congestion.	Incidence.
0.07	0.43	0.39	0.29
0.09	0.30	0.40	0.48
0.10	0.06	0.42	0.36
0.15	0.83	0.43	0.83
0.17	0.57	0.44	0.35
0.19	0.33	0.50	0.38
0.20	0.29	0.55	0.40
0.22	0.19	0.60	0.60
0.25	0.40	0.67	0.47
0.27	0.40	0.71	0.58
0.29	0.58	0.75	0.40
0.30	0.31	0.86	0.43
0.31	0.33	1.00	0.70
0.33	0.39	1.25	0.67
0.38	0.26	1.33	0.60

The coefficient of correlation is $+0.4324$. This result is biased because the effect of the size of the families has not been removed from this. In order to calculate the association between size and incidence the total number of persons belonging to different sizes was found out and the rates of incidence for respective groups were worked out after excluding the first cases from all the affected families. The table below gives the incidence as related to the size of the family.

TABLE XIX.

Distribution of the rate of incidence and size in the affected families.

Size.	Incidence.	Size.	Incidence.
2	0.50	10	0.30
3	0.38	11	0.37
4	0.41	12	0.35
5	0.29	13	0.35
6	0.53	15	0.50
7	0.46	16	0.33
8	0.36	18	0.60
9	0.25	20	0.11

The coefficient of correlation is -0.2209 .

In order to find out the independent or partial coefficient of correlation between congestion and incidence, and size and incidence another correlation coefficient has to be worked out between size and congestion.

TABLE XX.

Distribution of size and congestion in the affected families.

Size.	Congestion.	Size.	Congestion.
2	0.71	10	0.26
3	0.59	11	0.31
4	0.40	12	0.32
5	0.37	13	0.38
6	0.41	15	0.16
7	0.38	16	0.22
8	0.38	18	0.44
9	0.33	20	0.29

The coefficient of correlation is -0.6948 .

If the numbers 1, 2, and 3 denote respectively congestion, size, and incidence, we have,

$$r_{12} = -0.6948$$

$$r_{13} = +0.4324$$

$$r_{23} = -0.2209$$

The partial correlations obtained from these are:—

$$r_{13.2} = +0.3976$$

$$r_{23.1} = +0.1227$$

The value of $r_{13.2}$ ($+0.3976$) shows that as congestion diminishes, i.e., the number of rooms per person increases, the incidence also increases. This result goes against the contact infection theory according to which a high negative correlation ought to have been obtained.

SUMMARY.

1. Field investigations have been conducted in seven different centres in connection with outbreaks of the disease.

2. A set scheme for the collection of data has been followed and two schedules, one for the family and the other for the individual, so designed as to facilitate mechanical analysis, have been used for recording the information.

3. The main facts brought out by an analysis of the data are :—

- (a) Age is a determining factor in the incidence of the disease. Breast-fed infants always escape, children under five are rarely affected and beyond this age group, there is practically no difference in the incidence of the disease.
- (b) There is no difference of incidence in the two sexes.
- (c) Hindus and Mohammedans are equally liable to suffer.
- (d) Epidemic dropsy is chiefly disease of the middle class people.
- (e) Bengalees or those following their dietetic habits are most susceptible.
- (f) Factors common to the members of the family are more important in determining the incidence than merely the food habits (vegetarian or non-vegetarian) of the individual.
- (g) A striking fact in connection with the rice theory is that those who habitually reject the water in which rice is boiled are definitely more affected than those that use it. Probably economic status has something to do with this distribution.
- (h) No case is found amongst the non-consumers of mustard oil. Other things being equal the incidence increases with an increase in the quantity of mustard oil consumed.
- (i) As the number of rooms per person increases, the incidence also rises, a finding which goes against the contagion theory.

CONCLUSIONS.

1. The data presented failed to support either the rice theory or the contagion theory.

2. The observed facts could best be explained on the hypothesis that epidemic dropsy was due to a deleterious substance of unknown nature and origin, which might be present in certain supplies of mustard oil.

ACKNOWLEDGMENTS.

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APPENDIX I(a).

EPIDEMIC DROPSY INQUIRY, I. R. F. A.

Family Schedule.

I, II Village No.		III, IV House No.				V, VI Family No.						
Date of visit.		VII Fortnight of onset.										
Name of head of family												
Father's/Husband's name												
VIII Drinking water source		tank	well	tube-well		river.	lake		others			
		1	2	3		4	5		6			
IX Rice storage	Rice	freshly	husked	-3m	-6m	-1 year		over 1 year				
			0	1	2	3		4				
	Paddy			5	6	7		8				
X Source of rice	Selfgrown	Village shop										
	10		0	1	2	3	4	5	6	7	8	9
XI Other village	affected	unaffected			unknown							
	1	2			3							
XII Name of mill	Locality	affected			unaffected					unknown		
		0			1					2		
XIII Percentage of opaque grains	10	20	30	40	50	60	70	80	90	100		
	0	1	2	3	4	5	6	7	8	9		
XIV Method of storage of rice	1	2	3	4	5	6	7	8	9			
XV New rice (fortnight)												
XVI No. of persons in the family												
XVII Number of shop in village or ' Kulu ' house												
XVIII Shopkeeper's source of supply												
Visitors												
XIX When	-1wk	-2wk	-4wk	-2m	-5m	-6m	6m-					
	0	1	2	3	4	5	6					
	Locality	affected			unaffected					unknown		
		6			7					8		
Environmental conditions												
XX No. of rooms occupied by family												
XXI Sanitary condition good fair bad												
		1	2	3								
XXII Adults												
XXIII Children												
XXIV No. of cases of epidemic dropsy in the family within last one year												
XXV Sample	Rice	yes	no	Atap	milled	dhenki	Parboiled	milled	dhenki	Mustard oil	yes	no
		1	2	3	4		5	6			7	8
XXVI Economic status P. M. U. M. & R.												
		1	2	3								

Family Schedule Index.

I. Gourangdib, II. Patherkandi, III. Calcutta 'A', IV. Calcutta 'B', V. Karimganj,
VI. Sylhet, VII. Twenty-Tines, VIII. Jamshedpur.

-3m = less than 3 months; -6m = less than 6 months.

(1) Rice in *kuchuri* or similar containers (under damp condition)

- | | | | | | | | | | | | |
|-----|---|---|--------------|---|---------------|---|---|---|------|---|---|
| (2) | " | " | " | " | " | " | (| " | dry | " |) |
| (3) | Paddy | " | " | " | " | " | (| " | damp | " |) |
| (4) | " | " | " | " | " | " | (| " | dry | " |) |
| (5) | " | " | <i>morai</i> | " | <i>hamire</i> | | | | | | |
| (6) | Rice in gunny bags, earthenware pots, or similar containers (under damp conditions) | | | | | | | | | | |
| (7) | " | " | " | " | " | " | (| " | dry | " |) |
| (8) | Small quantities of rice at a time | | | | | | (| " | damp | " |) |
| (9) | " | " | " | " | " | " | (| " | dry | " |) |

XIX When: -1 wk = less than 1 week ago; -2 wk = less than 2 weeks ago; -4 wk = less than 4 weeks ago; -2m = less than 2 months ago; -5m = less than 5 months ago; -6m = less than 6 months ago; 6m- = more than 6 months ago.

APPENDIX I(b).

General Case Schedule.

I, II, III Serial No.				IV, V Village No.							VI, VII House No.						
VIII, IX Family No.				Investigator							X, XI Week of visit						
Name				Father's name													
				Husband's													
XII	Age	0-	1-	1-	3-	5-	15-	25-	35-	45-	55-	65-					
		0	1	2	3	4	5	6	7	8	9	10					
XIII	Sex	m	f	Marital status				s	m	w	Religion			h	m	e	o
		1	2					3	4	5				6	7	8	9
XIV	Caste	br	bd	kay	gow	sarak	bag	bau	mht	bania	dom	krmkr	mch	kulu	fshn	others	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
XV	Occupation	ll	ag	cul	spk	fh	ms	ml	art	med	ste	wm	swp	hwo	hwc	hwnc	beg
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		t ah c nk															
		16	17	18	19												
XVI	Health	well	indifferent	a-ill	ch-ill	Name of illness					c	case	doubtful	note a case			
		1	2	3	4							5	6	7			
XVII	Previous history of epidemic dropsy						-6m	6m-	-1y	-2y	-5y	-10y					
							1	2	3	4	5	6					
XVIII	Economic status	P.	M.	U. M. & R.													
		7	8	9													

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part III.

SOME LABORATORY STUDIES ON RICE AS THE ÆTIOLOGICAL AGENT.

BY

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SINCE the epidemiological data collected by us, at seven widely separated places where outbreaks of epidemic dropsy had occurred (*vide* Part II, this issue), failed to fit in with the various theories regarding the ætiology of the disease, we decided to put the different food theories to test by feeding human volunteers under controlled conditions. In selecting the material for our food experiments we had occasion to examine a few samples of rice bacteriologically and we soon discovered that Gram-positive spore-bearing proteolytic aerobes could be isolated from both the opaque and the non-opaque grains. This observation led us to investigate the matter a little more closely. We, therefore, repeated the experiments of other workers to see if the organisms of this type were especially abundant in the opaque parts of the grains with typical central or other types of opacities.

TABLE

Results of bacteriological examination of samples of rice using commercial formam as the disinfectant.

Sample number.	Local name of variety of rice.	Locality affected or unaffected.	Grade of rice and process of preparation from paddy.†	Storage.	Source and remarks.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND RESULTS.*		
								24 hours.	48 hours.	72 hours.
1	Beti	Unaffected	M. P. H.	1 month	{ From a family in Mymensingh	{ (a) Central (b) No opacity	{ Entire Split Entire Split	1/3	Not examined	3/3
								4/6	"	4/6
								0/3	"	3/3
								3/6	"	6/6
2	Balam	"	"	Not certain	{ From a market in south Calcutta.	{ (a) Central (b) No opacity	{ Entire Split Entire Split	1/3	"	3/3
								2/6	"	3/6
								1/3	"	3/3
								1/6	"	4/6
3.	?	Affected	M. S. H.	Fresh	{ From an affected family in Twenty-Lines.	{ (a) Central (b) No opacity	{ Entire Split Entire Split	0/3	0/3	0/3
								0/6	0/6	0/6
								0/3	0/3	0/3
								0/6	0/6	0/6

4	?	Unaffected	"	1 year	From Karimganj farm.	(a) Central (b) No opacity	Entire Split Entire Split	0/3 0/6 0/3 0/6	0/3 0/6 0/3 0/6	0/3 0/6 0/3 0/6
5	Mula	Affected	C. S. H.	3 weeks	From Hathi-khira Bazaar (Twenty-Lines).	(a) Central (b) No opacity	Entire Split Entire Split	0/3 0/6 0/3 0/6	1/3 0/6 0/3 1/6	1/3 0/6 0/3 1/6
6	?	"	"	"	From an affected family in Twenty-Lines.	(a) Central (b) No opacity	Entire Split Entire Split	0/3 1/6 0/3 1/6	0/3 2/6 0/3 1/6	0/3 2/6 0/3 1/6
8	Ropakatari	Unaffected	M. P. H.	Not certain	From a shop in Calcutta.	(a) Central (b) No opacity	Entire Split Entire Split	0/3 0/6 0/3 0/6	2/3 3/6 3/3 3/6	2/3 3/6 3/3 5/6

* In this and subsequent tables, the denominator denotes the total number of grains or half grains planted and the numerator, the number of those showing growth. Grains failing to give growth were kept under observation for seven days. These remarks also apply to results presented in subsequent tables.

† F=Fine, M=Medium, C=Coarse, P=Parboiled, S=Sun-dried, H=Hand-pounded, M=Milled.

TABLE I(a)—*concl'd.*

Sample number.	Local name of variety of rice.	Locality affected or unaffected.	Grade of rice and process of preparation from paddy.†	Storage.	Source and remarks.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND RESULTS.*		
								24 hours.	48 hours.	72 hours.
9	<i>Balam</i>	Unaffected	M. P. H.	Not certain	From a shop in Calcutta.	(a) Central	Entire Split	3/3 6/6	3/3 6/6	3/3 6/6
						(b) No opacity	Entire Split	2/3 3/6	3/3 6/6	3/3 6/6
						‡ (a) Central	Entire Split	1/3 3/6	3/3 6/6	3/3 6/6
						‡ (b) No opacity	Entire Split	0/3 1/6	3/3 6/6	3/3 6/6
10	"	"	M. P. H. (?)	"	"	(a) Central	Entire Split	2/3 6/6	3/3 6/6	3/3 6/6
						(b) No opacity	Entire Split	3/3 6/6	3/3 6/6	3/3 6/6
						‡ (a) Central	Entire Split	0/3 0/6	2/3 6/6	2/3 6/6
						‡ (b) No opacity	Entire Split	0/3 0/6	3/3 6/6	3/3 6/6

* In this and subsequent tables, the denominator denotes the total number of grains or half grains planted and the numerator, the number of those showing growth. Grains failing to give growth were kept under observation for seven days. These remarks also apply to results presented in subsequent tables.

† F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, M1 = Milled.

‡ In these examinations, the grains were steeped in formalin for 10 minutes instead of 5.

Note :—(1) Samples Nos. 3 and 6 were collected from affected families, and No. 1 from an unaffected family in an unaffected area. The rest of the samples were all collected from the bazaar.

(2) In Calcutta the information as to whether the locality was affected or not was obtained from the shopkeepers and some house-holders in the neighbourhood but no detailed investigation was carried out.

The samples were mostly obtained from the market and were mixtures of clear grains and grains with different types of opacities. Some of the samples, obtained from families in which cases of epidemic dropsy had occurred and also from families which had escaped from the disease during an epidemic in the village or the town, were included in these experiments. The examination consisted of:—

- (1) Naked-eye appearance of the grain for determining its variety and grade. Any obvious opacity present was also noted.
- (2) 'Water-test' to confirm the type of opacity.
- (3) Bacteriological examination of both clear and opaque grains from the same sample.

The usual technique (Pasricha *et al.*, 1936) for the isolation of organisms from rice practised at the School of Tropical Medicine, Calcutta, was employed. This consisted of:—

- (1) Steeping the grain in commercial formalin for five minutes to destroy the surface contamination.
- (2) Washing the grain repeatedly in sterilized distilled water or normal saline solution after removing it from the disinfectant.
- (3) Planting the whole rice grain as well as the cut surfaces of the grain on nutrient agar plates. The plates were incubated at 37°C. till growth appeared or for seven days if there was no growth. The results of these experiments are given in Table I(a) (see also note below).

In view of the fact that the results presented above were different from those reported by other workers, further experiments to test the efficacy of formalin as a disinfectant for the surface of the grains were performed in which all possible precautions were employed to eliminate any error in technique. The results are reported in Table I(b). Only whole grains free from macroscopic opacity were used for these experiments.

TABLE I(b).

Results of experiments to test the efficacy of formalin as a disinfectant for rice grains.

Serial number.	Local name.	Grade, method of husking and process of milling.*	Period of immersion into the disinfectant in minutes.	PERIOD OF OBSERVATION AND RESULTS.		
				24 hours.	48 hours.	72 hours.
1	Desi	M. P. Ml.	(a) 5	1/33	2/33	2/33
			(b) 10	0/31	0/31	0/31

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, Ml = Milled.

TABLE I(b)—*contd.*

Serial number.	Local name.	Grade, method of husking and process of milling.*	Period of immersion into the disinfectant in minutes.	PERIOD OF OBSERVATION AND RESULTS.		
				24 hours.	48 hours.	72 hours.
2	<i>Dadkani</i>	F. P. H.	(a) 5	2/44	4/44	5/44
			(b) 10	0/32	0/32	1/32
3	"	"	(a) 5	1/35	1/35	2/35
			(b) 10	0/26	0/26	2/26
4	<i>Balam</i>	M. P. H.	(a) 5	20/39	33/39	33/39
			(b) 10	4/30	12/30	15/30
5	<i>Desi</i>	"	(a) 5	0/28	0/28	0/28
			(b) 10	0/22	0/22	0/22
6	"	"	(a) 5	0/25	0/25	0/25
			(b) 10	0/28	0/28	0/28
7	"	"	(a) 5	0/23	0/23	0/23
			(b) 10	0/17	0/17	0/17
8	?	M. S. MI.	(a) 5	0/24	0/24	2/24
			(b) 10	0/21	0/21	0/21
9	?	"	(a) 5	0/19	0/19	0/19
			(b) 10	0/15	0/15	0/15
10	<i>Desi</i>	M. P. MI.	(a) 5	0/29	4/29	4/29
			(b) 10	0/31	0/31	0/31
11	"	"	(a) 5	0/45	0/45	0/45
			(b) 10	0/29	0/29	0/29

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, MI = Milled.

TABLE I(b)--contd.

Serial number.	Local name.	Grade, method of husking and process of milling.*	Period of immersion into the disinfectant in minutes.	PERIOD OF OBSERVATION AND RESULTS.		
				24 hours.	48 hours.	72 hours.
12	<i>Balam</i>	M. P. Ml.	(a) 5	19/19	19/19	..
			(b) 10	8/21	20/21	20/21
13	"	"	(a) 5	4/22	21/22	22/22
			(b) 10	1/29	5/29	25/29
14	<i>Desi</i>	"	(a) 5	0/28	0/28	0/28
			(b) 10	0/26	0/26	0/26
15	"	"	(a) 5	0/36	0/36	0/36
			(b) 10	0/34	0/34	0/34
16	<i>Balam</i>	"	(a) 5	4/35	30/35	35/35
			(b) 10	0/30	2/30	20/30
17	"	"	(a) 5	3/32	30/32	32/32
			(b) 10	0/31	25/31	30/31
18	"	"	(a) 5	0/46	6/46	40/46
			(b) 10	0/28	0/28	11/28
19	"	"	(a) 5	5/25	24/25	24/25
			(b) 10	0/30	5/30	26/30
20	"	"	(a) 5	20/25	25/25	25/25
			(b) 10	0/24	23/24	23/24
21	"	"	(a) 5	6/20	18/20	18/20
			(b) 10	0/33	8/33	10/33

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, Ml = Milled.

TABLE I(b)—*concl'd.*

Serial number.	Local name.	Grade, method of husking and process of milling.*	Period of immersion into the disinfectant in minutes.	PERIOD OF OBSERVATION AND RESULTS.		
				24 hours.	48 hours.	72 hours.
22	<i>Balam</i>	M. P. Ml.	(a) 5	20/25	25/25	..
			(b) 10	0/30	3/30	3/30
23	"	"	(a) 5	6/23	23/23	..
			(b) 10	0/25	15/25	15/25
24	"	"	(a) 5	0/31	28/31	28/31
			(b) 10	0/29	0/29	0/29
25	<i>Desi</i>	"	(a) 5	15/33	18/33	20/33
			(b) 10	0/30	0/30	1/30
26	"	"	(a) 5	2/38	2/38	2/38
			(b) 10	0/39	0/39	1/39
27	"	"	(a) 5	2/32	2/32	2/32
			(b) 10	0/38	0/38	1/38
28	"	"	(a) 5	0/27	0/27	0/27
			(b) 10	0/31	0/31	0/31
29	"	"	(a) 5	0/21	0/21	1/21
			(b) 10	0/26	0/26	0/26
30	"	"	(a) 5	0/26	1/26	1/26
			(b) 10	0/27	0/27	0/27

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, Ml = Milled.

Note.—The above samples of rice were obtained from different shops in Calcutta. The period of storage and epidemic history of the place were not ascertained.

Bose (1924) has drawn attention to the fact that efficient sterilization of the surface of the rice grain could be effected by using 1 in 1,000 solution of corrosive sublimate. Mercuric perchloride is the salt of a heavy bivalent metal with a strongly toxic cation and is a suitable disinfectant for our present purpose, because here we are mainly concerned with Gram-positive organisms, little or no protein is present to interfere with its action and no penetrating effect is desired. We, therefore, decided to repeat our experiments using this salt in the same dilution as employed by Bose, but adding 0.5 per cent of hydrochloric acid to make it more efficient. A set of parallel experiments with the two disinfectants were conducted at the same time using an equal number of grains in each instance. In both cases, plates were kept under observation for 72 hours or longer if there was no growth. The results are shown in Table II.

These experiments fully confirmed our previous observations as well as those of Bose. They showed that no growth could be obtained from inside the grain whatever type of opacity may be present, if surface growth could be eliminated.

However, mercuric perchloride, being a saline disinfectant, has a very low concentration coefficient (n being 3.8 in terms of Hg-ions). Before drawing final conclusions it, therefore, became necessary to test whether mercury was deposited on or absorbed by the surface of the grain in sufficient quantities to interfere with the subsequent growth of the organisms in the opaque interior of the grain even after the grain had been thoroughly washed.

TABLE II.

Comparative results of bacteriological examination of rice using formalin and mercuric perchloride as disinfectants.

Sample number.	Local name.	Locality affected or unaffected.	Grade of rice and process of preparation from paddy.*	Storage.	Source and remarks.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND RESULTS.					
								FORMALIN.			MERCURIC PERCHLORIDE.		
								24 hours.	48 hours.	72 hours.	24 hours.	48 hours.	72 hours.
1	Beli	Unaffected	M. P. H.	1 month	{ From a family in Mymensingh.	{ (a) Central (b) No opacity	{ Entire Split Entire Split	0/3	Not examined.	3/3	No growth.	No growth.	No growth.
								3/6	"	6/6	"	"	"
								1/3	"	3/3	"	"	"
								4/6	"	4/6	"	"	"
2	Balam	"	"	Not certain.	{ From a shop in Calcutta.	{ (a) Central (b) No opacity	{ Entire Split Entire Split	1/3	"	3/3	"	"	"
								1/6	"	4/6	"	"	"
								1/3	"	3/3	"	"	"
								2/6	"	3/6	"	"	"
9	"	"	"	"	{ "	{ (a) Central (b) No opacity	{ Entire Split Entire Split	3/3	3/3	3/3	"	"	"
								6/6	6/6	6/6	"	"	"
								2/3	3/3	3/3	"	"	"
								3/6	6/6	6/6	"	"	"
10	"	"	"	"	{ "	{ (a) Central (b) No opacity	{ Entire Split Entire Split	2/3	3/3	3/3	"	"	"
								6/6	6/6	6/6	"	"	"
								3/3	3/3	3/3	"	"	"
								6/6	6/6	6/6	"	"	"

11	"	"	M. P. Ml.	"	"	{ (a) Central (b) No opacity	{ Entire Split Entire Split	3/3 6/6 0/3 1/6	3/3 6/6 2/3 6/6	3/3 6/6 3/3 6/6	" " " "	" " " "	" " " "
12	Katarihog	"	F. P. H. (?)	"	"	{ (a) Central (b) No opacity	{ Entire Split Entire Split	0/3 1/6 0/3 0/6	1/3 1/6 1/3 1/6	1/3 2/6 2/3 2/6	" " " "	" " " "	" " " "
13	Mula	Affected	C. S. H.	3 weeks	From a family in Twenty-Lines.	{ (a) Central (b) No opacity	{ Entire Split Entire Split	0/3 1/6 0/3 1/6	0/3 1/6 0/3 1/6	0/3 1/6 0/3 1/6	" " " "	" " " "	" " " "
14	?	Unaffected	F. P. H. (?)	Not certain.	From a shop in Calcutta.	{ † (a) Central (b) No opacity	{ Entire Split Entire Split	0/3 4/6 1/3 1/6	1/3 5/6 3/3 2/6	2/3 5/6 3/3 3/6	" " " "	" " " "	" " " "
15	Katarihog	"	F. P. Ml.	"	"	{ (b) No opacity	{ Entire Split	0/3 0/6	0/3 0/6	0/3 0/6	" "	" "	" "
16	?	"	F. P. H.	"	"	{ (a) Central (b) No opacity	{ Entire Split Entire Split	0/3 0/6 0/3 0/6	Not examined. " " "	2/3 1/6 1/3 0/6	" " " "	" " " "	" " " "

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, Ml = Milled.

† The spore-bearing organisms isolated in these experiments were examined biochemically. The results are shown in Appendix. They closely resembled the reactions of the organisms, similarly isolated from the 'infected' or 'discussed' grains by other workers.

TABLE II—*conold.*

Sample number.	Local name.	Locality affected or unaffected.	Grade of rice and process of preparation from paddy.*	Storage.	Source and remarks.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND RESULTS.					
								FORMALIN.			MERCURIC PERCHLORIDE.		
								24 hours.	48 hours.	72 hours.	24 hours.	48 hours.	72 hours.
17	?	Unaffected	F. P. H.	Not certain.	From a shop in Calcutta.	(a) Central (b) No opacity	Entire Split Entire Split	0/3 0/6 0/3 0/6	Not examined. " " "	2/3 1/6 1/3 1/6	No growth. " " "	No growth. " " "	No growth. " " "
18	Balam	"	M. P. H. (?)	"	"	(a) Central (b) No opacity	Entire Split Entire Split	1/3 2/6 1/3 2/6	" " " "	3/3 6/6 3/3 6/6	" " " "	" " " "	" " " "
19	Kataribhog	"	F. P. Ml.	"	"	(b) No opacity	Entire Split	0/3 1/6	" "	0/3 3/6	" "	" "	" "
20	?	"	M. P. H.	"	"	(a) Central (b) No opacity	Entire Split Entire Split	0/3 1/6 0/3 1/6	1/3 3/6 3/3 4/6	1/3 3/6 3/3 4/6	" " " "	" " " "	" " " "
21	Kataribhog	"	F. P. Ml.	"	"	(b) No opacity	Entire Split	0/3 0/6	0/3 2/6	2/3 2/6	" "	" "	" "

* F = Fine, M = Medium, C = Coarse, P = Parboiled, S = Sun-dried, H = Hand-pounded, Ml = Milled.

† The spore-bearing organisms isolated in these experiments were examined biochemically. The results are shown in Appendix. They closely resembled the reactions of the organisms, similarly isolated from the 'infected' or 'disused' grains by other workers.

Note.—Sample No. 1 was collected from an unaffected family in an unaffected area and No. 13 from an unaffected family in an affected area. Rest of the samples were collected from the bazaar.

As a matter of fact, on testing with a saturated solution of sulphuretted hydrogen, a faint trace of mercury could be demonstrated on the surface of the grain even after a very thorough washing such as was carried out in our experiments. However, it was observed that at no stage in the experiment did the metal penetrate into the interior of the grain, but it remained confined only to a thin outer zone as demonstrated by sectioning the rice which had been treated with sulphuretted hydrogen before washing. To eliminate error arising out of the possible action of this trace of mercuric perchloride on the surface of the grain in interfering with the subsequent growth, from the opacity, due to its permeation to the centre of the grain, two types of experiments were devised as given below :—

(1) Before planting the entire or the split grains on the nutrient agar plate the surface of the grain was gently scraped to remove all traces of mercury. This operation did not make any difference in regard to the results previously obtained, as will be seen from Table III.

TABLE III.

Results of bacteriological examination of samples of rice using mercuric perchloride as the disinfectant. Scraped grain being used.

Sample number.	Type of opacity.	Entire or cut surface.	PERIOD OF OBSERVATION AND RESULT.		
			24 hours.	48 hours.	72 hours.
1	(b) No opacity {	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
2	(a) Central {	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
	(b) No opacity {	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
9	(a) Central {	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
	(b) No opacity {	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6

TABLE III—*concl.*

Sample number.	Type of opacity.	Entire or cut surface.	PERIOD OF OBSERVATION AND RESULT.		
			24 hours.	48 hours.	72 hours.
10	(a) Central	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
	(b) No opacity	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
20	(a) Central	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6
	(b) No opacity	Entire	0/3	0/3	0/3
		Split	0/6	0/6	0/6

(2) In these experiments both clear and opaque grains were used. A fine hole was bored in the centre of the grain. The bottom of the hole was carefully touched without coming in contact with the outer part of the hole, with the point of a fine needle, which had been previously contaminated with 'rice-bacillus', obtained through the courtesy of Dr. A. J. H. DeMonte of the School of Tropical Medicine, Calcutta. The opening was then sealed with paraffin-wax. These grains were then treated with mercuric perchloride, washed, and plated as before. The results are given in Table IV.

TABLE IV.

Results of bacteriological examination of rice artificially inoculated with 'rice-bacillus', the surface of the grain being subsequently sterilized with mercuric perchloride.

Sample number.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND RESULT.		
			24 hours.	48 hours.	72 hours.
1	No opacity	Entire	0/3	0/3	0/3
		Split	6/6	6/6	6/6

TABLE IV—*concl.*

Sample number.	Type of opacity.	Entire or split.	PERIOD OF OBSERVATION AND. RESULT.		
			24 hours.	48 hours.	72 hours.
2	(a) Central	Entire	0/3	2/3	2/3
		Split	6/6	6/6	6/6
	(b) No opacity	Entire	0/3	2/3	2/3
		Split	0/6	5/6	5/6
9	(a) Central	Entire	0/3	1/3	2/3
		Split	5/6	6/6	6/6
	(b) No opacity	Entire	0/3	1/3	1/3
		Split	3/6	6/6	6/6
10	(a) Central	Entire	0/3	0/3	0/3
		Split	4/6	5/6	6/6
	(b) No opacity	Entire	0/3	1/3	2/3
		Split	2/6	6/6	6/6
16	(a) Central	Entire	0/3	0/3	1/3
		Split	0/6	5/6	5/6
18	(a) Central	Entire	0/3	0/3	2/3
		Split	5/6	6/6	6/6
20	(b) No opacity	Entire	0/3	1/3	1/3
		Split	2/6	5/6	6/6

The non-permeation of the disinfectant in the interior of the grain is thus clearly demonstrated. In many instances growth was obtained from the whole ins also which was due to the leakage of the inoculum either through the bore crack in the grain, which might have developed at the time of washing or subsequent drying of the outer surface.

Gloster (1928) made sections of the so-called diseased grains and he failed find any evidence of liquefaction in the central opaque parts of the grains.

Hundreds of split grains were examined by us macroscopically and no evidence of liquefaction could be found. In a few samples infested with weevils (*Silvanus suranimensis**) the interior of the rice, which contained the beetle in all stages of development, was reduced to powder and the shell could easily be crushed between two fingers.

ARTIFICIAL PRODUCTION OF OPACITY.

It is stated that storage of rice under warm and damp conditions favours infection of grains with *B. vulgatus* and subsequent production of opacities in these grains. Experiments were conducted in which these conditions were imitated to see if opacities could be developed artificially in clear grains. Medium grade, parboiled, and milled grains which are believed to be most vulnerable to infection were employed. To further facilitate infection, saline emulsion of organisms, used for artificial inoculation in previous experiments, was sprinkled over the grains before storage. The grains were put in small open-mouthed earthen-ware pots and in small gunny bags.

These containers were placed on 4-inch thick layers of earth kept damp by frequent sprinkling of water, in a dark ill-ventilated room under the staircase. Similar containers, having unseeded rice, were also kept side by side with them, to serve as partial controls. Other samples of seeded and unseeded rice were placed in similar vessels and kept on the laboratory table. These experiments were conducted in August and September when conditions are said to be most favourable for the development of opacities. On examining the samples, kept in the dark room, after three weeks' storage, it was found that in all cases the grains in the lower portion of the containers had matted together and overgrown with fungi with considerable evolution of heat, but the upper layer had remained unaltered. No change was noticed, in the grains, kept on the laboratory bench, even after many months' storage, except that they became weevil eaten in course of time. Further experiments were conducted by placing seeded and unseeded rice, in sterile Petri dishes with wads of cotton-wool, moistened with water, placed in the centre. The dishes were kept in the incubator (at 37°C.) for 6 weeks but no opacities developed.

COLLECTION OF OPAQUE GRAINS FROM NON-EPIDEMIC AREAS.

In Part II of this series (this issue), attention has been drawn to the fact, that rice forms the staple article of diet in many parts of India but epidemic dropsy is confined to a comparatively small and restricted area. It was, therefore, thought that a study of rice obtained from non-epidemic dropsy areas will be of interest. Samples of clear grains and those showing typical central opacity were sent out in small capsules containing glycerine to different parts of India with the request that samples of rice containing a fair proportion of similarly opaque grains may be sent to us, together with the information about the prevalence of any disease resembling epidemic dropsy in the locality. In response to this request a number of samples was received from different districts of Madras. According to the statements received, there were no cases of epidemic dropsy present in the localities at the time. In fact, for all we know, the disease was unknown in these parts though

* Kindly identified for us by Dr. H. P. Chowdhury.

epidemics of beri-beri including the wet form had been reported from Guntur and Kistna Districts in 1929-30. The samples of rice, obtained from Madras, were subjected to macroscopic and bacteriological examinations.

Information regarding the presence and type of opacities is given in Table V. The results of bacteriological examination were very similar to those obtained with the Bengal samples. It is not considered necessary to reproduce them here.

TABLE V.

Samples of rice from various districts of Madras distributed according to the type of opacity and the percentage of opaque grains.

Serial number.	District.	CENTRAL OPACITY PER CENT.					MARGINAL OPACITY PER CENT.					Total samples.
		Nil.	1-25.	26-50.	51-75.	76-100.	Nil.	1-25.	26-50.	51-75.	76-100.	
1	Guntur ..	2	9	3	0	0	0	5	9	0	0	14
2	East Godavari	4	5	0	0	0	0	4	5	0	0	9
3	West Godavari	0	4	0	0	0	0	2	2	0	0	4
4	Nellore ..	9	4	0	0	2	2	6	2	1	4	15
5	Kistna ..	4	5	0	0	0	1	4	2	1	1	9
TOTAL ..		19	27	3	0	2	3	21	20	2	5	51

It is clear from the above table that the occurrence of the various types of opacities including the typical central opacity is not confined to regions where epidemic dropsy is prevalent.

CONCLUSIONS.

(1) The technique, so far described, for the surface sterilization of rice grains with formalin for demonstrating the infection in the central opacity failed, in our hands, to give the desired results in certain samples of rice.

(2) Growth from the surface of the grain could be effectively prevented by means of mercuric perchloride solution in 1 in 1,000 dilution, acidified with 0.5 per cent hydrochloric acid.

232 *Investigations into the Epidemiology of Epidemic Dropsy.*

(3) The traces of mercuric perchloride, left on the surface of the grain, in the process of disinfection, did not in any way interfere with the growth of the organism artificially introduced in the centre of the grain.

(4) The presence of the 'rice-bacilli' in the central or other types of opacities in the rice grain could not be demonstrated if surface contamination was effectively eliminated.

(5) Rice with high percentage of typical centrally opaque grains might be found in certain parts of Madras where epidemic dropsy does not occur.

(6) We failed to produce opacity in clear rice grains seeded with *B. vulgatus*, by storing them in a damp, warm, ill-ventilated cellar or in the incubator (at 37°C.) provided with sufficient humidity.

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APPENDIX.

Fermentation reactions of spore-forming aerobes isolated from rice.

Sample number.	Lactose.	Dulcitol.	Salicin.	Mannitol.	Maltose.	Glucose.	Saccharose.
1 (b)	—	—	—	—	A	A	A
2 (a)	—	—	A	A	A	A	A
2 (b)	—	—	A	A	A	A	A
14 (a)	—	—	—	A	A	A	A

See Table II for particulars regarding these samples.

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part IV.

EXPERIMENTS TO TEST THE VALIDITY OF INFECTION THEORY IN A SEMI-ISOLATED COMMUNITY.

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IN a number of outbreaks reported by different authors, cases have arisen in association with imported cases in villages or localities which were free from the disease. The course of events in such outbreaks suggested the transmission of the disease from one person to another by close contact or droplet infection. Many workers have supported this view, some of whom have been referred to in Part I of this series (this issue) to which reference may be made. Our own observations (see Part II, this issue) at Gourangdih were suggestive of the contagion theory. An opportunity to test the validity of this theory occurred after the conclusion of our first feeding experiment which will be described in a subsequent communication.

Towards the end of July 1936, information was received about the outbreak of epidemic dropsy amongst the Indian warders in a jail in Calcutta. There were 89 warders who resided in two barracks in the jail area, 43 occupying barrack No. I and 46, barrack No. II. They were derived from different communities

and formed into five distinct groups, each having different messing arrangements. As a rule members of the same group occupied contiguous beds (see Diagram 1

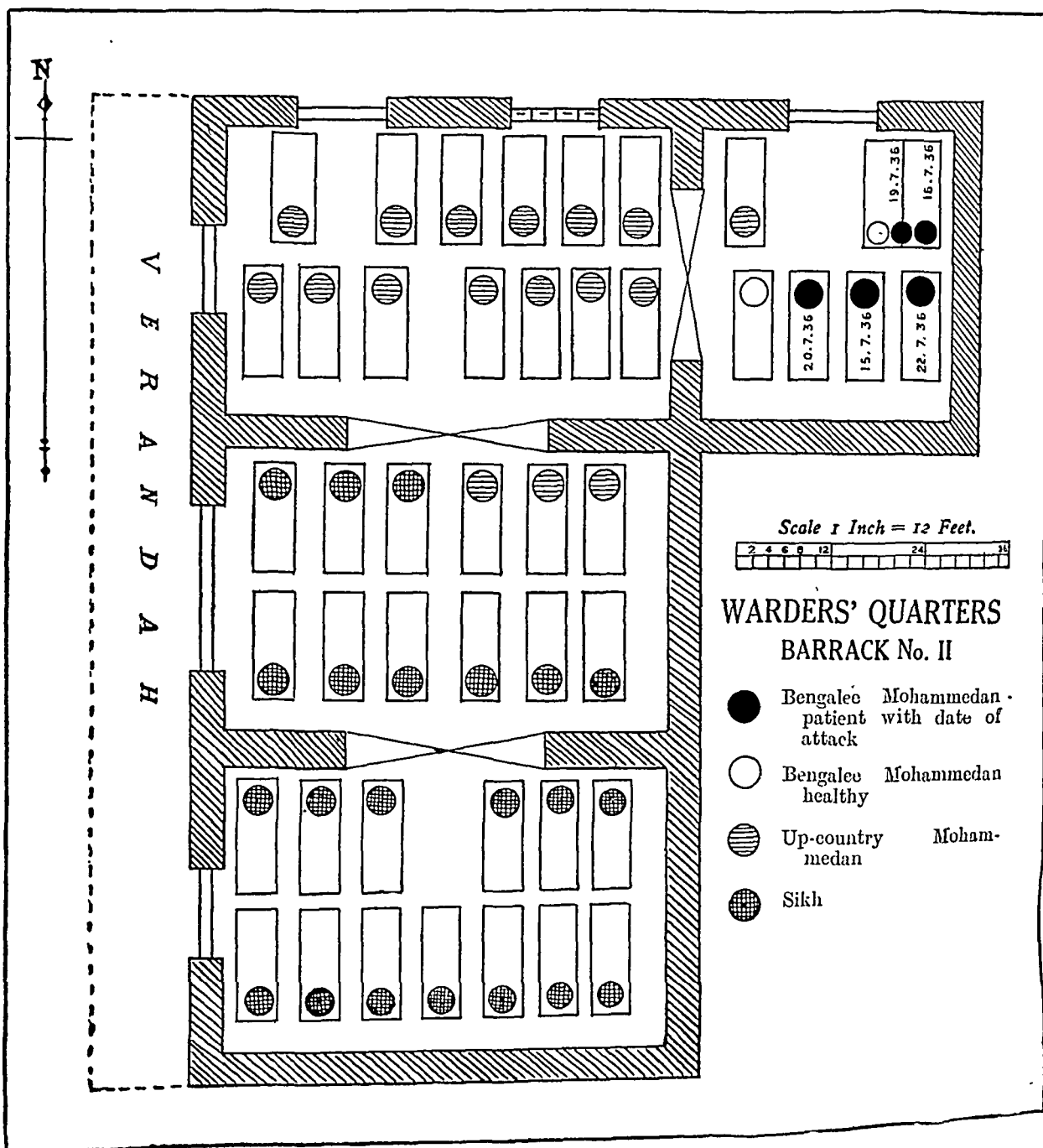


DIAGRAM 1.

for the distribution of the beds of the members of various groups in barrack No. II).

The messing arrangements of the different groups are shown in Table I.

TABLE I.

Messing arrangements of the various groups of warders.

Group.	Province of origin.	Religion.	Number in the group.	Sleeping place.	Messing place.
I.	Bengal	Hindu	7	Barrack No. I	Messing together in shed A.
II.	"	Muslim	7	Barrack No. II	Separate kitchen in shed A.
III.	Up-country (Bihar, U. P. and Punjab).	Hindu	34	Barrack No. I	Individual kitchen in shed A.
IV.	(a) "	Muslim	2	"	Own kitchen in shed A.
	(b) "	"	17	Barrack No. II	Messing together in shed B.
V.	Up-country (Punjab)	Sikh	22	"	Messing together in shed B.

The two kitchen sheds A and B were located near barracks Nos. I and II respectively. The different messes were partially separated from one another by partition walls.

The outbreak which started on the 15th July, 1936, had two striking features. It was confined to the Bengalee Mohammedans, five out of seven of whom became victims, and all the cases developed within a week. The order in which cases occurred is shown in Diagram 1. The arrangement of beds of the cases and the rapidity with which they developed would at first sight suggest droplet infection. However, strange though it seems, three men in this group slept in a common double bed during that part of the year and one of the men who escaped, shared the bed with the two others who developed the disease. Besides, the servant, who cooked for them but lived outside the jail area, was also attacked about the same time.

On the 24th July, two days after the development of the last case, the sites of the beds were re-allotted so that the beds of the patients alternated with those of healthy up-country Mohammedans. The distance between the adjacent beds varied between 12 and 18 inches and the passage between the beds was 2 feet (*vide* Diagram 2).

Proper arrangements were made to ensure that each individual slept in the bed allotted to him. The patients as well as their healthy neighbours were kept

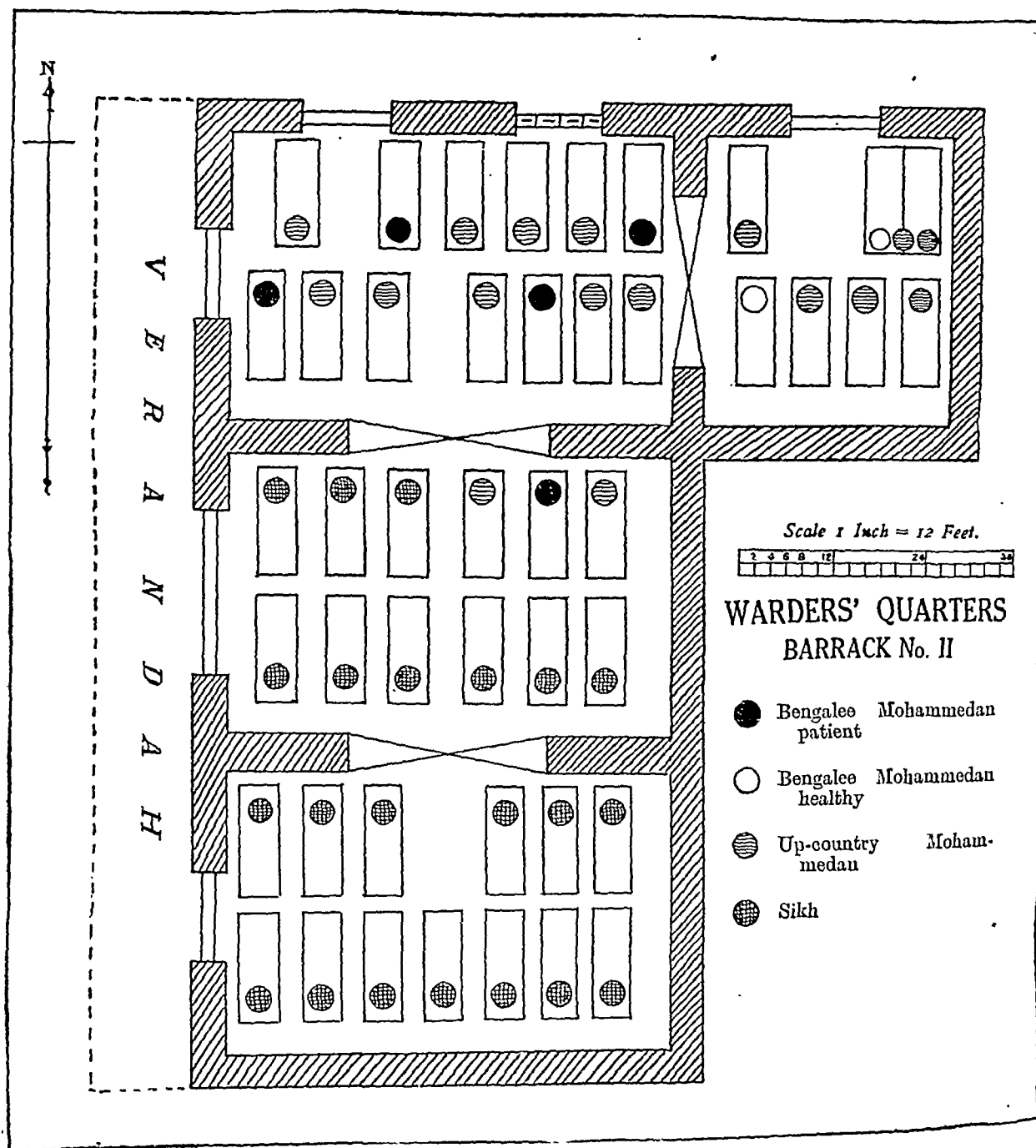


DIAGRAM 2.

under observation till the 18th August, 1936. Each individual was physically examined on alternate days. The other residents of the barrack were occasionally

examined for œdema of legs and frequent inquiries about the development of gastro-intestinal symptoms were made.

For a further period of three weeks, bi-weekly examination of the contacts was carried out, but no secondary cases were detected.

In connection with this epidemic we had a rare opportunity of studying the quantity and quality of food partaken, from day to day, by members of each group, as the accounts of daily purchases were carefully maintained by the secretaries of the different messes. A careful analysis of the articles of food consumed by the members of the different groups was made. A résumé of the findings is given below :—

GROUP I. Bengalee Hindus.

- (1) Rice for both meals.
- (2) Fish almost daily and mutton about once a month.
- (3) Mustard oil daily but practically no *ghee*.
- (4) Vegetables, *dals*, spices, etc.

GROUP II. Bengalee Mohammedans.

Practically the same diet as that of Bengalee Hindus.

GROUP III. Up-country Hindus.

- (1) Rice for midday meal, and *atta* and very rarely rice for the evening meal.
- (2) No fish or meat.
- (3) Small amounts of mustard oil and a fair quantity of *ghee*.
- (4) Vegetables, *dals*, spices, etc.

GROUP IV. Up-country Mohammedans.

- (1) Rice for the midday meal and *atta* for the evening meal.
- (2) Meat (mutton or beef), about 2 to 3 times a week and fish occasionally.
- (3) Fair quantities of mustard oil and variable amount of *ghee*.
- (4) Vegetables, *dals*, spices, etc.

GROUP V. Sikhs.

- (1) *Atta* for both meals.
- (2) No fish, but occasionally mutton.
- (3) No mustard oil, but plenty of *ghee*.
- (4) Vegetables, *dals*, spices, etc.

Since rice and mustard oil are of special importance in this connection, these two articles were closely studied. Main points are tabulated below.

The notes regarding rice refer to the stock in use at the time of investigation. In the case of Group II the supply of rice was obtained in bulk two weeks prior to the commencement of the outbreak. Vitamin-B₁ content of this sample was 39 International units per 100 g. which corresponded to the vitamin-B₁ content of partially polished rice. With regard to mustard oil, it was used only by Bengalee Hindus and Mohammedans in any quantity.

TABLE II.

Particulars about the rice and mustard oil consumed by the different groups.

Group.	Rice.	Mustard oil.
I.	Parboiled, milled, medium grade, 2 per cent of the grains showing central opacity. Fair growth of spore-formers on culture.	From the jail and rarely a day's supply from the bazaar. One ounce per head per day.
II.	Parboiled, coarse, milled, no grain showed typical central opacity but 10 per cent of the grains showed marginal opacity. Some grains were weevil eaten. On culture as before, copious growth of spore-formers.	From a shop in Kidderpore. One ounce per head per day.
III.	Parboiled, milled, medium grade, 18 per cent of the grains showing central or marginal opacity. No growth of spore-formers on culture. (This refers to the sample obtained from one kitchen with three members.)	From the jail and occasionally a day's supply from the bazaar. A very small quantity per head per day.
IV.	Parboiled, milled, mixed medium and fine, 10 per cent of the grains showed central opacity, badly weevil eaten. Fair growth of spore-formers on culture.	From the jail and occasionally a day's supply from the bazaar. About one-fourth ounce per head per day.
V.	No rice 	No oil.

The warders are expected to purchase the oil from the jail and they are allowed a concession of one anna per *seer*. The Bengalee Mohammedans were, therefore, at first unwilling to admit that they had been purchasing oil from the bazaar. The facts, however, came out on examination of their mess accounts. The jail oil is issued only on Tuesdays, while the account showed purchases made on other days and the rate charged was also less than the price payable by them at the jail. The servant on interrogation supplied the name of the shop from which he had been purchasing the oil. An attempt was made to trace the oil from the shop to the mill but it proved abortive.

CONCLUSIONS.

(1) No evidence in support of the contagion theory was obtained: However, in the absence of any knowledge of the period of infectiousness of the cases, if any, the possible method of spread, and immunity status of the contacts, the infection theory cannot be definitely discounted.

(2) The evidence points towards some article of diet consumed by the members of the affected group as being the causal factor. Only the fact that mustard oil was purchased from the bazaar for use in their mess is significant.

ACKNOWLEDGMENTS.

Our best thanks are due to Lieut.-Colonel M. A. Singh, I.M.S., for the facilities given to us for this investigation. We are also thankful to Dr. K. C. Das Gupta, Sub-Assistant Surgeon, for his hearty co-operation. Our thanks are also due to Dr. B. Ahmad for the analysis of the rice sample he made for us.

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part V.

FEEDING EXPERIMENTS ON HUMAN VOLUNTEERS.

BY

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IN Part I of this series we have given a historical survey of the literature on the epidemiology of epidemic dropsy and have shown that the ætiology of the disease is still shrouded in mystery and that the discussion mainly centres round three theories which have superseded other minor theories. The ætiological agents according to these three theories are:—

- (1) 'Diseased' rice,
- (2) Poisonous mustard oil, and
- (3) Infection communicable through close personal association with cases.

The rice theory does at present hold the field and forms the basis of administrative action, but none of the above theories can claim universal acceptance. In the eastern provinces, the disease frequently confronts public health workers as a major problem which becomes very vexatious in the absence of basic knowledge and definite scientific guidance. In attacking this problem, we have followed a long and tedious course which involves several stages that must necessarily be passed through in any systematic study. The historical survey was necessary

for the appreciation of the magnitude of the problem and of the arguments advanced by different workers placed under various circumstances. It provided us with a knowledge on which working hypotheses could be based that would form the background for further studies. The next stage was to observe in a precise manner, the events as they occurred in the field and the relevant facts which might help in seeking their explanation. These studies have been described in Part II of the series (this issue). They threw doubt on the validity of the rice theory, pointed out certain difficulties in accepting the contagion theory, and provided suggestive evidence for incriminating mustard oil as the probable ætiological agent. Thus, they served to narrow down the issues, but by themselves they could not offer positive evidence as to the nature of the ætiological agent. Each of these hypotheses remained to be tested under more controlled conditions. The significance of opacity in rice has been discussed in Part III of the series and it has been shown how difficult it is to accept the hypothesis that the opacity is due to the invasion of the rice grain by a spore-bearing ærobe. The negative results obtained on testing the contagion theory in a semi-isolated community have been discussed in Part IV of the series. In this communication we shall describe a series of feeding experiments on human volunteers, living under strictly controlled conditions. We believe that the results obtained confirm the tentative conclusions arrived at in our previous papers and throw a flood of light on this complicated problem. Altogether three experiments were conducted.

GENERAL SCHEME OF THE EXPERIMENTS.

(a) *Human subjects.*—Twelve volunteers were selected from amongst the convicts in Calcutta jails for each experiment. They were clinically examined, special attention being paid to the heart, lungs, bowels, blood, and urine. Those showing any detectable defects were rejected. They were promised richer and more delicious food in lieu of which they were required to give a written undertaking that they were willing to undergo the risks involved in the experiment, that they would not take any food except what was given to them by us for as long as the experiment lasted, and that they would subject themselves to medical examination when required to do so. They were allowed to perform their allotted duties but their movements were restricted so as to control their contact amongst themselves and with persons from outside. A watch was kept on new entrants to the jails, especially on those that were placed in the same barracks or workshops with the volunteers. There were no cases of epidemic dropsy in these jails at the time of the experiments.

(b) *Food.*—Three meals were served daily according to fixed schedules.

Provisions were daily received from the jail stores except rice and mustard oil which were supplied by us. Two varieties of rice were purchased in bulk. The 'healthy' rice was medium grade, parboiled, and milled, and the 'diseased' rice was also of the same type except that it had been stored for some time and had 40 per cent opaque grains. The 'healthy' rice yielded on culture few or no spore-bearing ærobes while the 'diseased' rice gave good growth.

Mustard oil was also of two kinds. One was the jail oil which was used at the time by the rest of the jail population and the other was the suspected oil which had been collected in the course of our investigations on epidemic dropsy in the

field. The two kinds of rice and the two kinds of mustard oil were stored separately in a special godown which was kept under lock and key, so that the stores could not be taken out except under the supervision of one of us. Vessels for taking out the rations bore special marks to prevent any possible mix up. Cooking was done under supervision in a separate kitchen, and all the necessary precautions were taken to ensure separate cooking of the two kinds of rice, as also of the curries, etc., with the two kinds of oil. Meals were also served under supervision and a note was made of the approximate amount of rice and mustard oil consumed by each prisoner. Any food left over was destroyed and the vessels were cleaned and properly stored under supervision. In all the three experiments, for purposes of special diet, the volunteers were divided into four groups of three each. Members of each group lived and worked together along with other prisoners but no two groups occupied the same barrack. The allotment of sleeping berths to each volunteer and his immediate neighbours was made before the commencement of the experiment and this arrangement continued throughout.

Experiment I.

As we have shown in a previous communication, cases of epidemic dropsy are most frequently seen amongst the middle-class Bengalee adults. Our subjects were, therefore, selected from amongst the educated *Bhadralog* class. They were more or less alike in their education and social habits and were employed as clerks or convict supervisors. They took keen interest in the experiment and intelligently co-operated with the investigators. With a view, however, to avoid the possibility of any errors arising out of the psychical effects, the exact nature of the differences in the special ingredients entering into the dietary of the control and the experimental groups was kept strictly confidential. Each individual was clinically examined before the commencement of the experiment, and found healthy. Malarial parasites were not found in the blood, differential count was normal, and the stools were free from parasites or eggs. The urine in all cases was free from sugar and albumin. The men had been in prison for at least three months and had had no history of sickness during this period. They were kept under observation for more than a month before the commencement of the experiment.

The suspected mustard oil.—About 85 ounces of oil was secured in its original container from an affected house at Karimganj. The evidence on which this brand of oil was suspected of being associated with epidemic dropsy may be briefly stated as follows :—

Karimganj had been free from epidemic dropsy for some months when a consignment of this particular brand of mustard oil (Gopal brand) was received from an oil mill in Calcutta by two wholesale provision stores 'A' and 'B'. The family of 'A's' proprietor resided in three different groups, viz., (1) group 'a' in the market area, (2) group 'a₁' in the village home at Karnamadhu, and (3) group 'a₂' in the residential area of the town. Groups 'a' and 'a₁' were each supplied with about 20 seers of 'Gopal' oil at about the same time, whereas group 'a₂', not being in need of more oil at that time, did not receive any, but was supplied with a different brand of oil at a later date. The first two groups were badly affected whereas the third completely escaped in spite of occasional close contact with the affected groups.

The family of 'B's' proprietor lived in two groups, one in their village home at Boran, and the other in the market area of Karimganj. Those living in the market area lodged in four different shops, but messed together. Both the groups used 'Gopal' oil which had been purchased in the original containers (about 20 seers each). Cases began to crop up in both groups simultaneously.

Apart from these grocers' families, none others were stricken with epidemic dropsy at Karnamadhu or at Boran. There were other instances in which isolated families were affected in different villages and in each case, history of purchase of 'Gopal brand' oil from the stores 'A' or 'B' at about the time referred to above could be traced. The head of the family, from which we obtained the supply of oil for this experiment, also kept a grocer's shop at Karimganj, but having run short of his stock he had purchased 'Gopal brand' oil in the original container from 'B's' shop for family use. Two branches of the family lived at Karimganj and the third resided in the village home at Bagabari. The consumption of this oil was simultaneously started by all the three branches of the family and all suffered from epidemic dropsy.

Controlled feeding.—Special food was given from the 25th May, 1936. The common schedule of diet for all the four groups was as follows:—

Early morning.

Rice	3 to 4 ounces per head.
Dal	1 ounce per head.
Mustard oil (jail product for all) ..		1/12 ounce per head.

Midday.

Rice	7 to 9 ounces per head.
Dal	2 ounces
Fish	1½ " "
Vegetables	4 " "
Mustard oil	½ ounce " "
Salt and spices as required.		

Evening.

Rice	6 to 8 ounces per head.
Dal	2 ounces " "
Meat or fish (3 times a week)	1½ " "
Vegetables	4 " "
Oil	½ ounce " "
Salt and spices as required.		

The only differences in the food of the four groups were with regard to the kind of rice and mustard oil used in the above schedules. Thus—

Group	I	received 'healthy' rice and jail-produced oil.
"	II	" " " " 'suspected' oil.
"	III	" " 'diseased' rice and jail-produced oil.
"	IV	" " " " 'suspected' oil.

On the 8th June, i.e., 14 days after the commencement of the special feeding, the supply of the suspected oil was exhausted. Each volunteer had, up to that date, consumed from 230 to 290 ounces of rice and 14 ounces of oil of the respective variety. No untoward symptoms were noticed in any of the subjects. The experiment, however, was continued for another week using oil of probably the same brand collected from another affected family. Each person had by this time consumed 21 ounces of oil. Only one volunteer X in group II complained of loss of appetite, burning sensation in the stomach, and slight constipation. These symptoms persisted for three days and the usual carminative mixture did him no good.

As only about 10 ounces of the 'suspected' oil were left, it was withheld from all the volunteers, except X who continued to take the oil for the next three days. At the end of this period he was attacked with the common cold, and showed a slight rise of temperature for a day, when his usual diet was stopped and milk was given to him instead. He was relieved of all his symptoms on the 20th June, 1936.

As the supply of 'suspected' oil was all exhausted, the experiment was conducted on six volunteers as before to see if the 'diseased' rice, when used for a long time, could produce the symptoms of epidemic dropsy. The other six were kept as controls. This continued up to the 8th July, 1936, when the experiment was concluded. At the end of the experiment and for more than three weeks after this, all the subjects were periodically examined and all of them were found to be in sound health.

The gastro-intestinal symptoms and the rise of temperature observed in volunteer X simulate early symptoms of epidemic dropsy but their correct interpretation is difficult. The total amount of 'suspected' oil ingested by him was 31 ounces, which, as we shall see later, should be quite sufficient to cause symptoms, if the oil was really potent. In any case the experiment should be considered as inconclusive.

Experiment II.

This experiment was an exact replica of experiment I, except that none of the subjects selected belonged to the *Bhadralog* class. The two varieties of rice employed were from the same stocks as used in experiment I, but the suspected oil was the one secured from a family in Jamshedpur during the outbreak described in Part II of the series. Four out of six members of the family had been stricken after consumption of the oil which had been purchased in the original container from the particular mill mentioned before. The first case in the family occurred after 13 days' use of the oil.

Here again the number of volunteers was 12. They were as before divided into four equal groups according to the kind of rice and mustard oil on which they were put. For convenience of description, the groups in this experiment bear the same numbers relative to the 'healthy' and 'diseased' rice and jail-produced and 'suspected' mustard oil, as in experiment I. The diet schedule in the two experiments was identical, except that a greater amount of oil (a little less than 2 ounces per head) was used in cooking. The special feeding commenced on the 22nd January, 1937, and continued uneventfully till the 26th, by which time each

subject had consumed 85 to 105 ounces of rice and $7\frac{1}{2}$ ounces of oil. On the 27th, three convicts, namely D* (II), B (IV), and C (IV), complained of abdominal uneasiness, fullness and acidity, and refused the early morning meal. They, however, took the midday and the evening meals though in slightly smaller quantities. Next day they made no complaints. On the 29th, however, all the subjects belonging to groups II and IV complained of loss of appetite, gurgling, fullness, and a vague sense of discomfort in the abdomen. They felt they did not have satisfactory motions and suggested an opening dose. All except A (IV) suffered from ill-defined pains all over the body, especially about the joints. Members of groups I and III on the other hand were all quite happy and enjoyed their meals. Œdema of the legs, pitting on pressure, appeared next evening in D (II), A (IV), and B (IV). While in the last two cases œdema was more or less confined to the legs and was equally developed on both sides, in D (II) it extended to the right foot and was definitely more developed on this side than on the other. Typical flushing of the affected parts was noticed in B (IV). Later the subjective symptoms became more marked in D (II) whose duties entailed hard physical labour. He complained of exhaustion on slight exertion and of his inability to carry on his work. All the members of groups II and IV except B (IV) felt feverish at night and F (II) was admitted to the hospital with a temperature of 103°F . on the 31st January. No malaria parasites could be detected in his blood next day, but he had had some quinine at night. No marked change in the knee-jerk of individual subjects was noticed. These developments had their effect on the morale of the volunteers and members of groups II and IV except A (IV) expressed their unwillingness to continue with the experiment. Later the nervousness spread to members of other groups. Even though they had no definite complaints some men of group III declined co-operation. (More detailed notes regarding each individual will be found in Appendix I.) All the subjects of the experiment were independently examined by Dr. R. N. Chowdhury, Dr. S. Sundar Rao, and Colonel R. N. Chopra, I.M.S., of the Calcutta School of Tropical Medicine. They confirmed our main clinical findings and agreed that the signs and symptoms in A (IV) and B (IV) had resulted from special feeding and that they resembled those seen in epidemic dropsy. A point of special interest is that A (IV), who unlike his compatriots continued to take the experimental diet till the 8th February and altogether consumed 27 ounces of 'suspected' mustard oil, had not entirely recovered from his illness till the 28th March when he was last seen. His weight was reduced from 108 lb. to 100 lb. and swelling appeared in the evenings after the day's work. The characteristic flush in the affected parts could also be observed. Dr. Refatullah of the Eye Infirmary who kindly examined the patient for us on the 21st March was unable to find any abnormality in the eyes. Regarding D (II) there was difference of opinion as to the nature and cause of his œdema because he was found to be a chronic filarial subject, the evidence of which had unfortunately missed our attention in the previous examination. Leaving this case out of consideration we come to the conclusion that a condition clinically resembling epidemic dropsy could be produced by the use of certain consignments

* The letters stand for individuals and the Roman figures in brackets refer to the group in which the subject was placed. This system has been used throughout.

of mustard oil either alone or in combination with 'diseased' rice. In order to clear up this point experiment III was conducted in which no 'diseased' rice was used.

It should, however, be noted that the 'suspected' oil used here was unadulterated in as much as it satisfied the requirements of the Bengal Food Adulteration Act of 1919. The analysis of a sample of the oil was kindly carried out for us by Dr. A. K. Sen and his report was as follows:—

Saponification value 174.7, iodine value 104.0. It does not contain any of the following, viz., (1) linseed oil, (2) sesame oil, (3) ground-nut oil, (4) castor oil, (5) mineral oil. It is genuine mustard oil.

Samples of mustard oil of the same brand collected from other affected families and used in experiment III were reported to be slightly adulterated.

Experiment III.

The experiment was designed a little differently from the other two. The total number of volunteers was the same, namely 12. All received 'healthy' rice from the same stock as was used in the previous experiments. The only variable factor was the mustard oil. The subjects were, therefore, divided for purposes of feeding into two groups instead of four, one receiving the 'suspected' oil and the other the jail product. With a view to prevent the spread of nervousness amongst the volunteers, each group was subdivided into two sub-groups of three each. Separate residential, working and feeding arrangements were made, so that one sub-group could not directly communicate with the other. Extra care was taken in the clinical examination before the commencement of the experiment and for this purpose the collaboration of Dr. R. N. Chowdhury was secured. The basic schedule of diet was the same as in previous experiments, but the following additions were made:—

1. Two ounces of uncooked pulped tomato with a dash of tamarind and unrefined sugar (*gur*) or two pieces of fresh lemon.
2. Fish twice every day.
3. Increase in mustard oil to $2\frac{1}{4}$ to $2\frac{1}{2}$ ounces per head.
4. As a change *chapati* was given in place of the morning meal three or four times.

Sub-groups I and II received the 'suspected' oil and sub-groups III and IV were given the jail product. To begin with, the 'suspected' oil was the same as used in experiment II, but after 12 days when this stock was exhausted, oil recovered from another affected family of Jamshedpur was substituted. Four out of ten members of this family had had the disease, the first case being developed 12 days after commencing the use of this oil.

Special feeding commenced on the 17th February, 1937. All the six volunteers in sub-groups I and II complained of loss of appetite on the 25th or 26th February, by which time they had consumed from about 19 to $21\frac{1}{2}$ ounces of the 'suspected' oil. A' (I), E' (II), and F' (II) further complained of unsatisfactory evacuation of bowels and other intestinal disturbances. C' (I), B' (I), and D' (II) felt feverish at night. On the 26th the last named developed hyperæmia and œdema on both sides which were more marked on the legs than on the

feet. Two days later he was admitted to the hospital with a temperature of 103°F. and cough, where, as a result of rest in bed, milk diet, and strong purgation, the œdema subsided but hyperæmia continued. His two comrades in the same sub-group were also admitted to the hospital on the 2nd March, one on account of bleeding from the gums and the other due to fever and burning sensation all over the body. Thereafter all the three gave up the experimental diet. However, E' (II) and F' (II) who were discharged from the hospital after one day's stay and had returned to their normal duties on convalescent diet developed œdema of the legs, eight and ten days later respectively, which persisted for over a fortnight. Of sub-group I, two members developed fever and bad cough. All three continued on the experimental diet though in smaller quantities till 13th March except for one man who missed it for four days when he was in hospital. They all developed œdema of the legs (*see* Plate VIII) between the 9th and the 12th March, and showed dilatation of heart. Later two of them suffered from bleeding gums. Œdema in all the six subjects was characterized by its almost complete disappearance in the mornings and reappearance in the evenings. Practically no œdema could be demonstrated after two or three days' complete rest in bed. Knee-jerk in all cases remained unaltered. Urine was sterile* on culture and was free from red blood cells and pus cells, but hyaline casts were found in the urine of five cases in two of whom granular casts and epithelial cells were also seen. There was, however, no albumin or sugar.

All the subjects were again kindly examined by Dr. R. N. Chowdhury on the 12th and by Lieut.-Colonel E. H. Vere Hodge, I.M.S., on the 16th†. Both of them confirmed the main clinical findings and declared them clinically identical with mild cases of epidemic dropsy. These views were also shared by Dr. K. V. Krishnan who jointly examined the cases with Lieut.-Colonel E. H. Vere Hodge, I.M.S. Dr. Refatullah who conducted a complete eye examination of all the subjects on the 21st March was unable to detect any abnormality.

The appreciation of the value of this experiment mainly rests on the clinical findings before and after the administration of the special diet and therefore abstracts of clinical notes together with other relevant information are given in Appendix II, where a brief statement of the ground covered in course of the clinical examination will also be found.

DISCUSSION.

Epidemic dropsy is rather an ill-defined clinical entity which closely resembles and may be easily confused with certain types of beri-beri. It is more than probable that the ætiological factors concerned in epidemic dropsy and beri-beri are entirely distinct. The main clinical features which the clinicians in Bengal rely upon for distinguishing epidemic dropsy from beri-beri are symptoms of gastrointestinal irritation, peculiar mottling of the skin and hæmorrhage from mucous surfaces. Progressive anæmia is another important sign. Absence of peripheral

* One or two samples of urine showed evident contamination with *Staphylococcus aureus* and *albus*.

† C' (I) who presented well-marked clinical syndrome (*see* Appendix II) was taken out of consideration by Lieut.-Colonel E. H. Vere Hodge, because he stated that he used to suffer from palpitation after hard work even before the experiment commenced. On physical examination the heart was found perfectly sound before the commencement of the experiment.

PLATE VIII.



Characteristic edema as seen in the experimental group.

the gastro-intestinal symptoms was five to six days. Œdema was delayed. The interval in this case was more variable being from 9 to 23 days. The field observations, more particularly at Jamshedpur, would seem to suggest almost the same period for the appearance of œdema. Having traced the oil, the public should be given relevant information about the consignment of oil which is responsible for the outbreak, so that they might discard its use. This action will not prevent the appearance of new cases, because people who have already taken sufficient quantity of the poisonous substance will necessarily be attacked, but removal of the causal factor will limit the size of the epidemic and perhaps increase the proportion of mild cases which would have become serious if continued use of the deleterious oil had been permitted.

SUMMARY.

(1) Three experiments on human volunteers living under controlled conditions designed to test the rôle of 'diseased' rice and 'suspected' mustard oil in the production of epidemic dropsy have been described.

(2) In one experiment practically no positive results were obtained. In the second experiment two, possibly three, subjects out of the six fed on another consignment of 'suspected' mustard oil developed signs and symptoms strongly suggestive of epidemic dropsy. In the two definite cases, the rôle of 'diseased' rice as a subsidiary ætiological factor could not be excluded. In the third experiment no 'diseased' rice was used, but all the six persons fed on 'suspected' mustard oil developed well-marked signs and symptoms characteristic of epidemic dropsy. None amongst the control groups, which were of the same size as the experimental ones, developed any symptoms.

(3) An interval of five to six days elapsed between the commencement of feeding and the development of gastro-intestinal symptoms. Œdema appeared after 9 to 23 days.

(4) Persons who took the deleterious oil for a short time made rapid recovery, but symptoms persisted for considerable time in those who used the oil over three weeks.

(5) Practical application of the results presented here is discussed.

CONCLUSION.

Evidence has been presented which strongly supports the conclusion that epidemic dropsy as commonly met with in epidemic form in Bengal, Bihar, and Assam is caused by the ingestion of a poisonous substance conveyed through the agency of mustard oil, the nature and origin of which is at present unknown.

ACKNOWLEDGMENTS.

It is now our pleasant duty to acknowledge our gratefulness to the gentlemen who have helped to bring the experiments to a successful conclusion. The present inquiries on epidemic dropsy owe their inception to Brevet-Colonel R. N. Chopra, C.I.E., K.H.P., I.M.S., and to him our best thanks are due for inviting us to

take part in the investigations. We tender our grateful thanks to Major-General D. P. Goil, K.H.P., I.M.S., for his helpful suggestions which have materially contributed towards the success of the investigation. We are thankful to Lieut.-Colonel R. E. Flowerdew, I.M.S., who very kindly obtained Government of Bengal's permission to carry out the experiments in Calcutta jails. But for the keen interest and co-operation of Lieut.-Colonel M. A. Singh, I.M.S., the then Superintendent of the Presidency Jail, we could have made little headway with our investigations. Later as Officiating Inspector-General of Prisons, he further helped us in arranging the experiments in another prison and we take this opportunity of expressing our best thanks to him. We are also grateful to Lieut.-Colonel S. L. Patney, I.M.S., and Lieut.-Colonel M. Das, I.M.S., for their courtesy, co-operation, and helpful suggestions. Of the other members of the jail staff who helped us in our work, Dr. S. C. Chowdhury, Dr. M. M. Barory and Jamadar Warder of the Central Jail deserve special mention. We are especially grateful to our colleagues Brevet-Colonel R. N. Chopra, C.I.E., K.H.P., I.M.S., Lieut.-Colonel E. H. Vere Hodge, I.M.S., Dr. K. V. Krishnan, Dr. S. Sundar Rao, and Dr. Refatullah for taking the trouble of making clinical examinations of the volunteers. Dr. R. N. Chowdhury, who was jointly responsible for selecting the subjects in experiment III and who carried out repeated examinations, deserves our special thanks. We are glad to acknowledge the loyal services of Dr. A. C. Das Gupta.

APPENDIX I.

Examination of individual volunteers.

(A) EXPERIMENTAL GROUP :—

A (IV—'diseased' rice and 'suspected' mustard oil), well-developed, hard-working and bright Hindu male, 27 years, weaver, been in jail for two years. No previous illness and complaints since in jail. Height 5 ft. 1½ in., weight 108 lb. Pulse 62.

Total amount of special food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 256 ounces ; oil : 26½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms :—

29-1-37. Discomfort in the abdomen, gurgling and diminished appetite after ingestion of 12½ ounces of oil and 146 ounces of rice.

31-1-37. Fever at night, giddiness, gurgling in the abdomen and disinclination for work. Towards the end of the experiment, the appetite had greatly diminished and he took about a third of the food he used to take before the commencement of the experiment.

Physical signs :—

General appearance : dull and very much reduced.

Weight : 19-1-37, 108 lb. ; 31-1-37, 107 lb. ; 7-2-37, 108 lb. ; 27-3-37, 100 lb.

30-1-37. Slight oedema of both legs was noticed after consuming 17½ ounces of oil and 181½ of rice. It disappeared next day and appeared again after about one week. One month later he again developed the oedema and was then admitted to hospital.

31-1-37. Slight erythema of both legs.

8-2-37. Heart : nothing abnormal found. Pulse 66.

B (IV—'diseased' rice and 'suspected' mustard oil), healthy-looking, active Hindu male, 25 years, weaver, been in the jail for ten months, no previous illness, no complaint since in jail. Height 5 ft. 4 in., weight 98 lb. Pulse 60.

Total amount of special food consumed :—

Dates : 22-1-37 to 2-2-37 ; rice : 193 ounces ; oil : 20½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms :—

24-2-37. Diminution of appetite after the ingestion of 7½ ounces of oil and 100 ounces of rice.

29-1-37. Vague discomfort, fullness and gurgling in the abdomen.

30-1-37. Pain in the whole body, sore throat and cough.

31-1-37. Incomplete evacuation of the bowels and weakness. Towards the end of the experiment, the appetite diminished to less than half.

Physical signs :—

General appearance : dull and sickly.

Weight : 19-1-37, 101 lb. ; 31-1-37, 101 lb. ; 7-2-37, 101 lb.

30-1-37. Oedema of both legs with slight flush in the evening after consuming 165 ounces of rice and 17½ ounces of oil. It was present for four days after which it disappeared, but the flush continued for a few days more.

8-2-37. Heart : apex just internal to the nipple line ; sounds normal. Pulse 62 per minute. Blood pressure 130/85 mm. of Hg.

C (IV—'diseased' rice and 'suspected' mustard oil), healthy-looking, hard-working Hindu male, 29 years, weaver, been in jail for six months, dysentery in infancy and occasional orchitis,

no complaints since in jail except dysentery 3 months back. Height 4 ft. 10½ in., weight 99 lb. Pulse 62.

Total amount of special food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 239½ ounces ; oil : 22½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms :—

27-1-37. Diminished appetite and discomfort in the abdomen after ingesting 105 ounces of rice and 7½ ounces of oil.

28-1-37. Fever at night.

29-1-37. Pain in the body specially in the epigastrium, gurgling, fullness and vague discomfort in the abdomen.

2-2-37. Slight orchitis developed in the left side (admitted to hospital).

Physical signs :—

General appearance : not much reduced.

Weight : 19-1-37, 99 lb. ; 31-1-37, 101 lb. ; 7-2-37, 101 lb. No œdema noticed.

2-2-37. Orchitis developed in the left side.

8-2-37. Heart : no abnormality found. Pulse 66.

D (II—' healthy ' rice and ' suspected ' mustard oil), moderately-developed, active and bright Hindu male, 30 years, coir pounder, been in the jail for three months, smallpox in infancy, no complaints since in jail. Height 5 ft. 1½ in., weight 92 lb. Pulse 72. Slight deformity of the spine.

Total amount of special food consumed :—

Dates : 22-1-37 to 4-2-37 ; rice : 207½ ounces ; oil : 22½ ounces.

Signs and symptoms after the commencement of the experiment :—

Subjective symptoms :—

27-1-37. Abdominal discomfort, fullness and acidity, loss of appetite, after the ingestion of 105 ounces of rice and 7½ ounces of oil.

29-1-37. Exhaustion after slight exertion, gurgling and vague discomfort in the abdomen and pain in the body.

30-1-37. Fever at night and pain in the whole body.

4-2-37. Incomplete evacuation of the bowels.

11-2-37. Fever (admitted to hospital).

Physical signs :—

General appearance : dull and sickly.

Weight : 19-1-37, 92 lb. ; 31-1-37, 94 lb. ; 7-2-37, 95 lb.

30-1-37. Œdema of legs especially marked in the right after consuming 166 ounces of rice and 17½ ounces of oil. Later it became marked and continued up to 12-2-37 and then disappeared. After about three weeks, the œdema was noticed again and he was admitted to hospital.

8-2-37. Heart : apex in the mammary line in the 5th space. Pulse 80 per minute. Blood pressure 100/65 mm. of Hg.

Glands : Inguinal glands of the right side found enlarged (no evidence of recent involvement).

11-2-37. Fever, temperature 100.2°F.

29-3-37. No œdema (discharged from hospital).

E (II—' healthy ' rice and ' suspected ' oil), moderately-developed, active Hindu male, 28 years, coir pounder, been in the jail for 3 months, no previous illness except fever 10 years back, no complaint since in jail. Height 5 ft. 1½ in., weight 91 lb. Pulse 68.

Total amount of special food consumed :—

Dates : 22-1-37 to 4-2-37 ; rice : 208 ounces ; oil 22½ ounces.

Signs and symptoms after the commencement of the feeding :—

Subjective symptoms :—

27-1-37. Appetite diminished after the ingestion of 100½ ounces of rice and 7½ ounces of oil.

252 *Investigations into the Epidemiology of Epidemic Dropsy.*

29-1-37. Fullness, gurgling and discomfort in the abdomen and pain in the body.

30-1-37. Incomplete evacuation of the bowels, fever at night, frequency of micturition at night and marked loss of appetite.

Physical signs :—

General appearance : not much changed.

Weight : 19-1-37, 91 lb. ; 31-1-37, 95 lb. ; 7-2-37, 94 lb.

No œdema of feet or legs noticed.

Heart : nothing abnormal found. Pulse 74 per minute.

F (II—‘ healthy ’ rice and ‘ suspected ’ oil), healthy-looking, active Hindu male, 27 years, coir pounder, been in jail for 11 months, no previous illness except fever one year back, no complaint since in jail except fever 6 months ago with slight enlargement of the spleen. Height 5 ft. 3 in., weight 94 lb. Pulse 74.

Total amount of special food consumed :—

Dates : 22-1-37 to 2-2-37, missed one day (1-2-37) when in hospital ; rice : 185½ ounces ; oil : 19½ ounces.

Signs and symptoms after the commencement of the feeding :—

Subjective symptoms :—

27-1-37. Fullness of the abdomen and diminished appetite after ingestion of 105 ounces of rice and 7½ ounces of oil.

29-1-37. Incomplete evacuation of the bowels, fullness, discomfort and gurgling in the abdomen and pain all over the body.

31-1-37. Fever at night, exhaustion after slight exertion and pain in the abdomen and calf muscles.

1-2-37. Fever, vomiting and headache (admitted to hospital). Fever came down next day.

Physical signs :—

General appearance : slightly reduced and dull.

Weight : 19-1-37, 94 lb. ; 31-1-37, 98 lb. ; 7-2-37, 96 lb.

No œdema of the leg or feet was noticed.

Heart : nothing abnormal found. Pulse 84.

1-2-37. Temperature 103°F. (no malaria parasites seen in the blood).

G (III—‘ diseased ’ rice and jail oil), healthy, active Hindu male, 31 years, weaver, been in jail for 2½ years, no previous illness, no complaint since in jail except slight fever 1½ years back. Height 5 ft. 3 in., weight 106 lb. Pulse 76.

Total amount of food consumed :—

Dates : 22-1-37 to 4-2-37 ; rice : 243½ ounces ; oil 28½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil, except slight cough and weakness on 3-2-37.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 106 lb. ; 31-1-37, 108 lb. ; 7-2-37, 107 lb.

H (III—‘ diseased ’ rice and jail oil), well-developed, hard-working Hindu male, 30 years, cloth weaver, been in jail for 14 months, no previous illness, no complaint since in jail except an ulcer in the tongue. Height 5 ft. 5 in., weight 117 lb. Pulse 70.

Total amount of food consumed :—

Dates : 22-1-37 to 4-2-37 ; rice : 272½ ounces ; oil : 27½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil, except aggravation of the ulcer tongue.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 117 lb. ; 7-2-37, 118 lb.

I (III—'diseased' rice and jail oil), well-developed, healthy, active Mohammedan male, 32 years, cloth weaver, been in this jail for two years, no previous illness, no complaint since in this jail except occasional attack of cold. Height 5 ft. 5 in., weight 129 lb. Pulse 64.

Total amount of food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 347 ounces ; oil : 34½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 129 lb. ; 31-1-37, 133 lb. ; 7-2-37, 133 lb.

(B) CONTROL GROUP :—

J (I—'healthy' rice and jail oil), well-developed, active Hindu male, 29 years, bearer, been in the jail for one and a half years, no previous illness, except fever ten years back, no complaint since in this jail. Height 5 ft. 5½ in., weight 110 lb.

Total amount of food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 369½ ounces ; oil 34½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 110 lb. ; 31-1-37, 114 lb. ; 7-2-37, 112 lb.

K (I—'healthy' rice and jail oil), well-developed, active and bright Hindu male, 35 years, clerk, been in the jail for 9 months, typhoid 8 years back, no complaint since in this jail, height 5 ft. 5 in., weight 123 lb.

Total amount of food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 352½ ounces ; oil : 34½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 123 lb. ; 31-1-37, 127 lb. ; 7-2-37, 129 lb.

L (I—'healthy' rice and jail oil), rather poorly-developed but active Mohammedan male, 40 years, bearer, been in jail for 20 months, no previous illness except occasional fever, no complaint since in this jail except occasional attack of common cold. Height 5 ft. 3½ in., weight 97 lb. Pulse 68.

Total amount of food consumed :—

Dates : 22-1-37 to 8-2-37 ; rice : 300 ounces ; oil : 34½ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weight : 19-1-37, 97 lb. ; 31-1-37, 101 lb. ; 7-2-37, 103 lb.

APPENDIX II.

The ground covered during the course of examinations.

(a) Clinical history during the term in jail and previous to conviction.

(b) Complaints, if any, and their duration (special attention was paid to appetite and ability to undergo hard physical exertion).

(c) Work and residence in jail.

(d) Physical examination :—

- (1) General appearance.
- (2) Height and weight.
- (3) Eyes.
- (4) Tongue, palate, throat, gums, and teeth.
- (5) Neck veins and glands.
- (6) Pulse and condition of the heart.
- (7) Blood pressure (Dr. Chowdhury).
- (8) Abdomen including liver, spleen, bowels, etc.
- (9) Inguinal and femoral glands.
- (10) Scrotum.
- (11) Legs for œdema.
- (12) Knee-jerks.
- (13) Hyperæsthesia in the calf muscles.

Besides these, a general examination of all the systems was made.

(e) Laboratory examinations :—

- (1) Stool for parasites or their ova and abundance or otherwise of Gram-positive bacilli.
- (2) Urine for specific gravity, reaction, sugar, and albumin.
- (3) Blood for filaria and malaria parasites and differential white cell count.

Examination of individual volunteers.

For the sake of brevity the negative findings are omitted and mention is made only of such signs and symptoms as have direct bearing on the disease.

(A) EXPERIMENTAL GROUP :—

A' (I), rather poorly-developed but active and bright Mohammedan male, 20 years, weaver, been in the jail 9 months, dysentery about 10 years back, used to get occasional pain in the right testicle about one year ago, no complaints since in jail, except poor appetite occasionally. Height 4 ft. 10½ in., weight 84 lb. Pulse 70 per minute. Blood pressure 115/78 mm. of Hg.

A few small soft glands palpable in the groin.

Total amount of special food consumed :—

Dates : 17th February to 13th March, missed 4 days from the 3rd when in hospital ; rice : 211 ounces ; oil : 37½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—

- 24-2-37. Indigestion and constipation after ingestion of 16½ ounces of oil.
- 26-2-37. Abdominal fullness, unsatisfactory evacuation of bowels, loss of appetite, quantity of food taken was gradually reduced to one-third or less after two weeks.
- 28-2-37. Vomiting tendency, pain around the umbilicus, small liquid motions.
- 1-3-37. Pain in the calf muscles, tingling of feet and legs, distressing cough, and epigastric pain.
- 2-3-37. Complained of fever at night.

These symptoms persisted for some time but later extreme sense of weakness and exhaustion was added on, which became the predominant symptom.

Physical signs :—

General appearance : dull, seedy, and much reduced.

Weight : 16-2-37, 84 lb. ; 12-3-37, 81 lb. ; 30-3-37, 80 lb. Temperature 100°F. on 3rd morning, variable thereafter, not rising above 100°F.

4-3-37. Tongue coated.

12-3-37. Slight œdema of both legs by which date 37 ounces of oil had been consumed. Œdema marked later.

16-3-37. Heart : slightly dilated ; foetal type of rhythm heard (Col. Vere Hodge). Pulse 80. Blood pressure 110/70 mm. of Hg.

Laboratory examinations :—

Blood : total R. B. C. count, 17-3-37, 2·83 millions per c.mm. ; 30-3-37, 2 millions per c.mm.

Stool : definite increase in Gram-positive bacilli.

Urine : a few hyaline casts present.

B' (I), well-developed, active and bright Mohammedan male, 26 years, weaver, been in the jail for 6 months, no previous illness, no complaints since in the jail except slight rawness of the tongue and ringworm on the scrotum. Height 5 ft. 8 in., weight 129 lb. A faint systolic bruit (Dr. Chowdhury) in the mitral area. Pulse 78 per minute. Blood pressure 114/75 mm. of Hg.

Total amount of special food consumed :—

Dates : 17th February to 14th March ; rice : 278 ounces ; oil : 45½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—

25-2-37. Fever at night after ingestion of 19 ounces of oil.

26-2-37. Loss of appetite, quantity of rice taken gradually decreased from 26th February onwards to less than half towards the end of the experiment.

1-3-37. Pain in joints and calf muscles. Unsatisfactory evacuation of bowels.

These symptoms persisted for some time, later on weakness and disinclination for work were the chief complaints.

Physical signs :—

General appearance : dull and sickly.

Weight : 16-2-37, 129 lb. ; 12-3-37, 129 lb. ; 30-3-37, 128 lb.

10-3-37. Œdema of legs and ankles by which date 42½ ounces of oil had been consumed.

12-3-37. Œdema of the legs became very marked with slight flush.

16-3-37. Heart : fairly dilated, marked systolic murmur in the mitral area. Pulse 82 per minute. Blood pressure 110/70 mm. of Hg.

25-3-37. Bleeding from the gums, which lasted 4 days.

Laboratory examinations :—

Blood : total R. B. C. count, 17-3-37, 3·88 millions per c.mm. ; 30-3-37, 3·00 millions per c.mm.

Stool : definite increase in Gram-positive bacilli.

Urine : a few hyaline and granular casts present, also a few epithelial cells.

C' (I), fairly well-developed, active and bright Mohammedan male, 26 years, weaver, been in the jail for 4 months, smallpox in childhood, no complaints since in jail except small ulcer on the right side of the tongue and palpitation of the heart after very hard work. Had also cough 3 days before the commencement of the experiment. Height 5 ft. 1½ in., weight 105 lb. Pulse 62 per minute. Blood pressure 130/85 mm. of Hg.

A few small and soft glands palpable in the groin.

Total amount of special food consumed :—

Dates : 17th February to 12th March ; rice : 243 ounces ; oil : 43½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—

21-2-37. Condition of the ulcer tongue aggravated.

23-2-37. Distressing cough.

25-2-37. Fever after ingestion of 19 ounces of oil.

4-3-37. Vomiting, fever, marked loss of appetite, quantity of food taken gradually decreased to less than a third towards the end of the experiment, abdominal fullness, pain around the umbilicus, incomplete evacuation of the bowels, and slight diarrhœa.

25-3-37. Pain in defæcation with slight bleeding.

Physical signs :—

General appearance : dull and much reduced.

Weight : 16-2-37, 105 lb. ; 12-3-37, 103 lb. ; 30-3-37, 99 lb.

3-3-37. Tongue coated.

256 *Investigations into the Epidemiology of Epidemic Dropsy.*

9-3-37. Swelling of legs after consuming 40½ ounces of oil.

13-3-37. Marked œdema of legs (admitted to hospital).

19-3-37. No demonstrable œdema (discharged from hospital).

23-3-37. Typical flush and marked œdema of both legs (admitted to hospital). Fairly severe bleeding from a verrugoid growth in the upper gum and also slightly per rectum.

16-3-37. Heart: dilated. Pulse 80. Blood pressure 122/80 mm. of Hg.

Laboratory examinations:—

Blood: total R. B. C. count, 17-3-37, 3.07 millions per c.mm.; 30-3-37, 2.08 millions per c.mm.

Stool: definite increase of Gram-positive bacilli.

Urine: a few hyaline casts present.

D' (II), rather poorly-developed, fairly active but not very bright Mohammedan male, 20 years, cane worker, been in the jail for 2 years, no previous illness, no complaints since in jail except dysentery 6 months ago, eye trouble 3 months ago and slight rawness of the tongue. Height 5 ft. 5 in., weight 91 lb. Pulse 70 per minute. Blood pressure 118/70 mm. of Hg.

Slight folliculitis of the skin of the extremities and the trunk.

Total amount of special food consumed:—

Dates: 17th February to 27th February; rice: 159 ounces; oil: 26½ ounces.

Signs and symptoms after commencement of feeding:—

Subjective symptoms:—

23-2-37. Condition of the ulcer of the tongue aggravated.

25-2-37. Fever at night after the ingestion of 19 ounces of oil.

26-2-37. Incomplete evacuation of the bowels, gurgling in the abdomen, cough, and loss of appetite.

28-2-37. Fever (admitted to hospital), diarrhœa and pain during defæcation.

Physical signs:—

General appearance: run down and sickly.

Weight: 16-2-37, 91 lb.; 12-3-37, 98 lb.; 30-3-37, 91 lb.

Temperature: 28-2-37, 103°F.

26-2-37. Slight œdema and flush in both the legs, by which date 24 ounces of oil had been consumed.

6-3-37. Œdema marked.

16-3-37. Heart: no abnormality found. Pulse 84. Blood pressure 115/70 mm. of Hg.

Laboratory examinations:—

Blood: total R. B. C. count, 18-3-37, 2.79 millions per c.mm.; 30-3-37, 2.50 millions per c.mm.

Stool: Gram-positive bacilli fairly increased.

Urine: a few hyaline and granular casts present, also a few epithelial cells.

E' (II), well-developed, active and bright Mohammedan male, 24 years, cane worker, been in the jail for 6 months, no previous illness, no complaints since in jail except occasional pain in the knee and elbow joints, frequency of micturition at night and a small ulcer at the tip of the tongue. Height 5 ft. 2½ in., weight 120 lb. Pulse 80 per minute. Blood pressure 135/80 mm. of Hg.

Total amount of special food consumed:—

Dates: 17th February to 1st March, rice: 168½ ounces; oil: 29 ounces.

Signs and symptoms after commencement of feeding:—

Subjective symptoms:—

23-2-37. Condition of the ulcer of the tongue aggravated.

24-2-37. Burning and itching sensation all over the body.

26-2-37. Incomplete evacuation of the bowels, loss of appetite, pain around the umbilicus after ingestion of 21½ ounces of oil.

- 28-2-37. Gurgling in the abdomen and diarrhoea.
- 1-3-37. Fever at night and pain all over the body.
- 4-3-37. Heaviness of both legs towards the evening.

Physical signs :—

General appearance : pulled down and reduced.

Weight : 16-2-37, 120 lb. ; 12-3-37, 118 lb. ; 30-3-37, 116 lb.

4-3-37. Slight ulceration of the tongue.

11-3-37. Slight œdema of the legs and feet especially around the ankles, gradually increased especially towards evening. 29½ ounces of oil were ingested before the appearance of œdema, which was marked later.

16-3-37. Heart : no abnormality. Pulse 88. Blood pressure 130/90 mm. of Hg.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 4.35 millions per c.mm. ; 30-3-37, 3.47 millions per c.mm.

Stool : definite increase in Gram-positive bacilli.

Urine : a few hyaline cast present.

F' (II), well-developed, hard-working and bright Hindu male, 26 years, cane worker, been in the jail for 8 months, no previous illness except fever two years ago, no complaints since in jail except occasional acidity, ulcer tongue and high coloured urine. Height 5 ft. 1½ in., weight 124 lb. Pulse 80 per minute. Blood pressure 138/90 mm. of Hg.

Total amount of special food consumed :—

Dates : 17th February to 1st March ; rice : 183 ounces ; oil : 29½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—

20-2-37. Condition of the ulcer of the tongue aggravated.

25-2-37. Fever at night, incomplete evacuation of the bowels with several thin stools after ingestion of 19 ounces of oil.

26-2-37. Loss of appetite and vomiting tendency.

27-2-37. Loose motions but no satisfactory evacuation.

1-3-37. Pain in the throat and bleeding from the gums.

7-3-37. Burning sensation and heaviness of both legs and feet towards the evening.

Physical signs :—

General appearance : dull and much reduced.

Weight : 16-2-37, 124 lb. ; 12-3-37, 118 lb. ; 30-3-37, 119 lb.

20-2-37. Ulceration of the tongue.

12-3-37. Slight œdema of the legs after consuming 29½ ounces of oil. Distinct later.

16-3-37. Heart : no abnormality found. Pulse 84 per minute.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 4.26 millions per c.mm. ; 30-3-37, 2.84 millions per c.mm.

Stool : Gram-positive bacilli definitely increased.

(B) CONTROL GROUP.

G' (III), well-developed, active and bright Hindu male, 26 years, worker in the printing press, been in the jail for one year. No previous illness, no complaint since in jail except ulcer of the tongue one month ago. Height 5 ft. 4 in., weight 100 lb. Pulse 72 per minute. Blood pressure 135/85 mm. of Hg.

Total amount of food consumed :—

Dates : 17th February to 16th March ; rice : 455½ ounces ; oil : 59 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : nil.

J, MR

258 *Investigations into the Epidemiology of Epidemic Dropsy.*

Physical signs :—

General appearance : no change.

Weights : 16-2-37, 100 lb. ; 12-3-37, 101 lb. ; 30-3-37, 98 lb.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 4.17 millions per c.mm. ; 30-3-37, 4.35 millions per c.mm.

Stool : slight increase in Gram-positive bacilli.

H' (III), well-developed, hard-working Mohammedan male, 24 years, worker in the printing press, been in the jail for 4 months. No previous illness, no complaints since in jail except occasional slight constipation and pain in the abdomen if bowels not moved. Height 5 ft. 3½ in., weight 124 lb. Pulse 80. Blood pressure 130/90 mm. of Hg.

Total amount of food consumed :—

Dates : 17th February to 12th March ; rice : 394 ounces, oil : 52 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weights : 16-2-37, 114 lb. ; 12-3-37, 114 lb. ; 30-3-37, 112 lb.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 3.79 millions per c.mm. ; 30-3-37, 3.92 millions per c.mm.

I' (III), fairly well-developed, active and bright Hindu male, 24 years, worker in the printing press, been in the jail for 8 months. No previous illness, no complaints since in jail except slight stomatitis and congestion of throat. Height 5 ft. 2 in., weight 108 lb. Pulse 76 per minute. Blood pressure 122/75 mm. of Hg.

Total amount of food consumed :—

Dates : 17th February to 16th March ; rice : 467 ounces ; oil : 59 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : Nil.

Physical signs :—

General appearance : no change.

Weight : 16-2-37, 108 lb. ; 12-3-37, 106 lb. ; 30-3-37, 105 lb.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 4.32 millions per c.mm. ; 30-3-37, 4.00 millions per c.mm.

Stool : slight increase in Gram-positive bacilli.

J' (IV), well-developed, hard-working Mohammedan male, 28 years, worker in the printing press, been in the jail for 2 months. Dysentery in his infancy, no complaint since in jail except rawness of the tongue and enlarged tonsil. Pulse 72 per minute. Blood pressure 120/80 mm. of Hg.

Total amount of food consumed :—

Dates : 17th February to 16th March ; rice : 514 ounces ; oil : 59 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weights : 16-2-37, 138 lb. ; 12-3-37, 137 lb. ; 30-3-37, 136 lb.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 3.65 millions per c.mm. ; 30-3-37, 4.61 millions per c.mm.

K' (IV), well-developed, active Mohammedan male, 22 years, worker in the printing press, been in the jail for 8 months. No previous illness, no complaint since in jail except folliculitis in the legs. Height 5 ft. 1½ in., weight 117 lb. Pulse 70. Blood pressure 144/100 mm. of Hg.

Total amount of food consumed :—

Dates : 17th February to 16th March ; rice : 600 ounces ; oil : 58½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : nil except fever on 12-3-37 for one day only.

Physical signs :—

General appearance : no change.

Weights : 16-2-37, 117 lb. ; 12-3-37, 118 lb. ; 30-3-37, 118 lb.

12-3-37. Temperature 101.4°F.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 2.79 millions per c.mm. ; 30-3-37, 3.05 millions per c.mm.

L' (IV), well-developed, active Mohammedan male, 26 years, worker in the printing press, been in the jail for 8 months. No previous illness, no complaint since in jail except slight rawness of the tongue and enlargement of the left testicle. Height 5 ft. 1½ in., weight 114 lb. Pulse 72 per minute.

Total amount of food consumed :—

Dates : 17th February to 16th March ; rice : 456 ounces ; oil : 59½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms : nil.

Physical signs :—

General appearance : no change.

Weights : 16-2-37, 114 lb. ; 12-3-37, 114 lb. ; 30-3-37, 113 lb.

Laboratory examinations :—

Blood : total R. B. C. count, 18-3-37, 5.02 millions per c.mm. ; 30-3-37, 4.40 millions per c.mm.

STUDIES ON THE AETIOLOGY OF EPIDEMIC DROPSY.

EFFECT OF PLASMA ON TISSUE CULTURE AND CHORIO-ALLANTOIC MEMBRANES OF THE CHICK.

BY

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FOLLOWING the lead indicated by the pioneer work of Acton and Chopra (1925) it is still held that a histamine-like substance is responsible for the symptoms met with in epidemic dropsy. While further clinical and pharmacological studies (Acton and Chopra, *loc. cit.*; Chopra and De, 1937) justify the rôle of this basic substance in producing the symptoms, epidemiological observations strongly suggest that the primary causative factor might be something else. The way in which the disease is produced and propagated leads one to consider the possibility that the causative agent may be some living organism capable of propagation and infection which possibly is responsible for producing the toxins. The biochemical studies carried out in this Laboratory (Chopra, Mukherjee and Gupta, 1935) have made it clear that, in the blood serum of patients suffering from epidemic dropsy, the pseudo-globulin fraction is considerably increased, while the albumin fraction is diminished to a great extent. While to this diminution of albumin clinical symptoms, such as œdema, have been ascribed, there seems to be an indication for the belief that this increased pseudo-globulin may be associated with some antitoxin formation (Pasricha *et al.*, 1936). This, no doubt, lends an indirect support to the causation of the disease by some living organism but further evidence must be forthcoming before any definite conclusion can be arrived at. We, therefore, tried to study the problem from different points of view and in course of our investigations we thought it proper to study the effect of the plasma from persons attacked with the disease on tissues cultivated *in vitro* in the hope that it might furnish some clue as to the nature of the causative factor. The details of the work and the results obtained therefrom constitute the subject-matter of the present paper.

EXPERIMENTS AND RESULTS.

(a) *Collection of the plasma.*—Plasma was separated by centrifugalization of the blood drawn from properly diagnosed epidemic-dropsy patients, the whole operation of bleeding, separation of the plasma, and its preservation being carried out in the cold with aseptic precautions. It was incidently observed that, unlike the plasma from the normal blood, the plasma from epidemic-dropsy patients presented a certain degree of opacity and that the blood from which it was obtained was, in many cases, relatively unstable as compared with the blood from normal subjects. This was shown by the fact that the plasma could not be separated from the blood from normal subjects without centrifugalization in the cold, while in epidemic-dropsy cases it separated out spontaneously merely on keeping the blood undisturbed in the cold for some time. This has been observed particularly in the blood of some of the acute cases of epidemic dropsy of the virulent type.

(b) *Tissue-culture experiments.*—Tissue-culture experiments were carried out in hanging drop preparations on cover-slips. The explant was made by tissues obtained from chicks' embryo suspended in a mixture of the extract derived from the same embryo and of the homologous plasma obtained from a cock. The details of the method have been presented in an earlier paper (Chopra, Das and Mukherjee, 1936). In the present case tissues for growth were obtained from the heart, the liver, and the spleen of the embryo.

To study the effect of the toxin on the tissue growth the explant was treated with a drop of the plasma obtained from epidemic-dropsy patients and allowed to grow. Controls were kept by adding a drop of the plasma from normal human subjects on another explant from the same tissue in another set of cover-slips. For each specimen of the plasma, tissues from at least four different embryos were tried and for each separate embryo a dozen explants were made. In all, therefore, a specimen of plasma from any patient was tested in a large number of different explants and normal controls were kept for each of them. More than 50 patients suffering from epidemic dropsy were studied in this connection.

Our results indicate that the toxic action was not very marked in the case of the liver and the spleen tissues. It is only with the heart tissue that our experiments met with some success. The most striking effect that was evident at first sight was the gradual inhibition of the rhythmic throbbing, bringing about a total stoppage in 72 to 90 hours. In the controls, however, throbbing continued up to the 13th or 14th day uninterruptedly. Tables I and II show how the rate varies from that of controls and how it gradually slows down.

The second effect observed was that in the explants treated with epidemic-dropsy plasma, the heart tissue showed a less vigorous growth than the controls and the individual cells in the former were not so healthy as in the latter. This chequered growth did not continue for long but stopped much earlier (in seven days' time) than in the controls where they survived up to the 17th day. For further investigations we observed some of these preparations under the microscope both in stained and unstained conditions. Almost every case of inhibition of tissue growth was found to be associated with a simultaneous fatty degeneration in the cells which was evident both in stained and unstained specimens. Microphotographs showing such degeneration together with photographs of corresponding

normal controls have been presented in support of our contention. A close inspection will reveal a number of small fat globules appearing in the specimen incubated with epidemic-dropsy plasma.

While examining the specimens with different stains we tried the effect of a few vital stains such as Nile blue and neutral red in concentrations of 1 in 50,000 on these explants. It was found that certain cytoplasmic changes of granular nature had occurred in some of the cells of a few explants (*vide* microphotographs and camera lucida drawings in Plate IX), where inhibition of tissue growth and degenerative changes were also present. Cells from normal controls, however, never showed such cytoplasmic changes.

It must be mentioned in this connection that these responses, viz., the stoppage of throbbing, the inhibition of tissue growth, and the appearance of cytoplasmic granules, could be observed only in the explants treated with plasma from fresh and acute cases of epidemic dropsy and were not marked with plasma from chronic cases. The cytoplasmic changes could be detected in only 25 per cent of our cases, while the stoppage of throbbing was more general occurring in as much as 90 per cent of the cases.

Our results in general undoubtedly go to establish the presence of some toxin in the plasma from epidemic-dropsy patients.

A further group of experiments was carried out, an inoculum from a cultured explant showing the cytoplasmic and other changes being transferred to new explants in series. Positive results were obtained up to the fifth passage wherein all the phenomena, viz., stoppage of the heart beat, stoppage of tissue growth, and the appearance of cytoplasmic granules were induced in some cases. These results might suggest the possibility of the presence of an infective agent rather than a chemical toxin.

(c) *Experiments on the chorio-allantoic membrane of eggs.*—As a next step in our investigations we tried to observe the effect of the inoculation of the epidemic-dropsy material on the chorio-allantoic membrane. The following technique was employed :—

Eggs were incubated for ten to twelve days and properly candled. The junction of the embryo and the sac was then marked out. The external surfaces of the eggs were sterilized and the shell removed to expose the inner shell membrane, which was then punctured and the epidemic-dropsy plasma dropped in with the help of a sterilized pipette. The fluid was automatically sucked in. The opening was then carefully sealed by sterile molten paraffin and the egg was again incubated for five or six days. At the end of this period it was opened at the small end and the whole embryo taken out. The allantoic membrane was carefully spread on a sterile Petri dish and watched for any characteristic lesions. A set of four eggs was inoculated for each specimen of epidemic-dropsy plasma, and the plasma from more than 60 cases were studied in the course of our experiments.

In about 20 per cent of our cases white macroscopic patches were observed which numbered two or three in each egg. These were similar to those observed by Brandly (1935) with *Laryngotracheitis* virus but were smaller in size and fewer in number than he has described.

EXPLANATION OF PLATE IX.

- Fig. 1. Microphotographs of living tissue cultures after 60 hours. (a) Normal control, (b) tissue treated with epidemic-dropsy plasma. (Magnification approximately $\times 100$.) In (b) cells have undergone degenerative changes.
- „ 2. Microphotographs of tissue cultures stained with iron-hæmatoxylin after 6 days' incubation. (a) Normal control, (b) tissue treated with epidemic-dropsy plasma. (Magnification approximately $\times 100$.) Degenerative changes are very marked in (b).
- „ 3. Microphotographs of living tissue culture. (Magnification approximately $\times 900$.) Degenerative changes showing granules inside the cells.
- „ 4. Microphotographs of tissue culture stained by Giemsa slow staining process. (Magnification approximately $\times 1,000$.) Growing cells containing cytoplasmic granules.
- „ 5. Camera lucida drawing of normal fibroblastic cells taken from a tissue culture.
- „ 6. Camera lucida drawing of fibroblastic cells of tissue culture treated with epidemic-dropsy plasma showing cytoplasmic inclusions.



Fig. 1 (a).



Fig 1 (b).



Fig. 3.



Fig. 2 (a).



Fig. 2 (b).

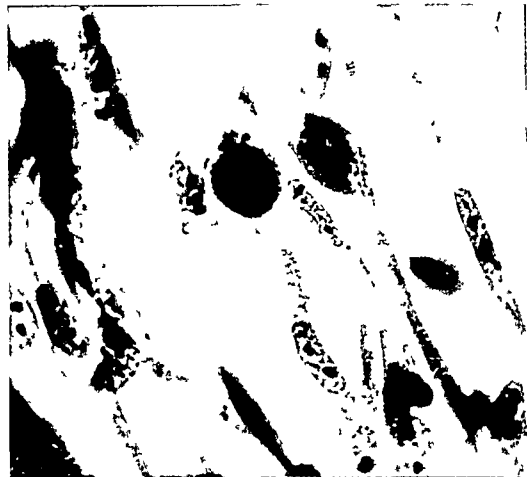


Fig. 4.

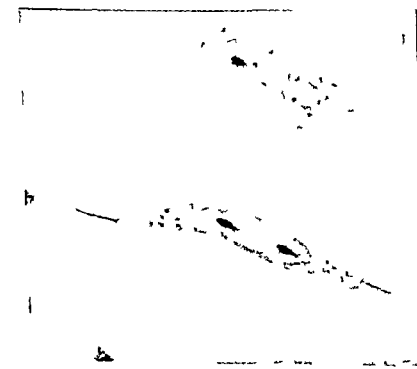


Fig. 5.



Fig. 6.

Lesions were cut out and macerated with quartz and filtered through Seitz filter. The filtrate was again used as an inoculum for the next passage. Up to the fourth passage the lesions were marked but in the fifth passage they exhibited a tendency to disappear. In the sixth passage these lesions could not be observed in any case.

DISCUSSION.

The results of our experiments indicate the presence of an agent in the plasma of cases of epidemic dropsy which is capable of causing degenerative changes in living cells maintained in tissue culture. The transmissibility in series of these changes and the lesion produced in the chorio-allantoic membrane of the chick suggest a resemblance to the action of certain filtrable viruses, but the responses obtained both in experiments in tissue culture and on the chorio-allantoic membrane were milder in character than those obtained in the case of many well-known viruses. They were not transmissible indefinitely and only occurred in a certain percentage of cases. It is interesting to note that the response with heart tissue was marked, while no response occurred with liver and spleen tissue of the embryo. A possible relationship of the finding to the common incidence of heart symptoms in epidemic dropsy (Chopra, Choudhury and De, 1937) is suggested although it cannot be stated that these phenomena are definitely correlated.

The nature of the cytoplasmic granules found in the cells is not yet determined but these show certain resemblances to the granules produced in tissue cultures by the virus of *Laryngotracheitis* of birds. The presence of a toxic agent in the plasma of epidemic dropsy cases can be considered to be established but the nature of this agent will, especially in view of the results obtained in serial transmission, require further investigation.

TABLE I.*

CONTROL EXPERIMENTS.

Showing the rate of throbbing of heart tissue in explants treated with plasma from normal subjects.

Number.	Initial beats per minute.	Rate after 24 hours.	Rate after 48 hours.	Rate after 72 hours.	Rate after 7 days.	Rate after 10 days.
1	82	60	60	55	50	50
2	65	60	55	50	45	45
3	68	64	60	55	50	45
4	68	62	55	50	45	40
5	72	60	52	48	42	38
6	74	62	50	48	40	32

* A large number of cases were actually worked out but for the sake of brevity a few cases only have been presented in the table.

TABLE II.*

EXPERIMENTS WITH EPIDEMIC-DROPSY PLASMA.

Showing the rate of throbbing of the heart tissue in explants treated with plasma from epidemic-dropsy patients and incubated at 37.4°C.

Number.	Initial beats per minute.	Rate after 24 hours.	Rate after 48 hours.	Rate after 72 hours.	Rate after 4 days.
1	80	60	38	32	Stopped.
2	82	60	36	21	"
3	80	65	34	20	"
4	84	66	28	16	"
5	88	65	30	18	"
6	82	62	35	16	"
7	84	66	27	12	"
8	80	65	24	20	"
9	78	64	28	10	"
10	84	60	30	Stopped.	"
11	76	50	21	"	"
12	80	60	24	10	"

* A large number of cases were actually worked out but for the sake of brevity a few cases only have been presented in the table.

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A STUDY OF OSSIFICATION AS OBSERVED IN INDIAN SUBJECTS.

BY

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IN the course of routine work in radiology as Honorary Radiologist to the Medical College Hospitals, Calcutta, I was struck by the difference in the times of union of epiphyses with diaphyses in Indian hospital patients as compared with European subjects of similar age. Having in many cases to express an opinion on age as determined by *x*-ray findings in Courts of Law, I commenced, in 1928, an investigation on the ossification in Indian and Anglo-Indian girls. This work was confined to the elbow, wrist, and hand. The results were published in a paper in the April number of the *Indian Medical Gazette*, 1930.

This present work was begun shortly after. Several difficulties have been encountered. Subjects for examination of known age have not been easy to obtain. All cases where the age was at all doubtful have been rigidly excluded. The average hospital patient has been quite useless for this work, owing to the uncertainty of age—it being a curious fact that many such patients in this country simply do not know how old they are.

Prior to the adoption of radiological methods, the only way of determining the times of appearance of fusion of bony centres was by means of macerating the bones concerned. The statistics obtained were necessarily limited.

Stevenson (1924) used the method to study the processes of ossification in 110 skeletons. His paper contains some very extraordinary observations discrediting radiological methods. He describes skiagrams as 'merely a confusing medley of shadows'. The findings of Pryor (1928), which he attempted to discount, to the effect that union of epiphyses takes place considerably earlier in girls than in boys have been amply confirmed by subsequent observers, notably Paterson (1929), Parsons and Davies (1926), and Flecker (1932*a* and *b*). The work of these radiologists has been of the utmost importance, providing accurate standards of comparison in England in the case of Paterson, Parsons and Davies, and Australia in the case of Flecker.

The study of the figures published by Pryor, Paterson, and Flecker, in addition to those in this series, conclusively prove that in the vast majority of cases earlier ossification takes place in girls. Such is even noted in the appearance of centres, though it is more marked in the union of epiphyses.

Adair and Scammon (1921) have shown that even in the foetus ossification takes place more rapidly in females.

The earlier bony development in females is, after all, to be expected in view of the earlier onset of puberty in this sex. There is every reason to believe that increased activity of the anterior lobe of the pituitary body and the thyroid gland near puberty influence ossification to some extent; many cases have been reported of sexual precocity associated with the early fusion of epiphyses. The paediatrist is frequently faced with such patients.

The suppression of the activity of the hormones of the anterior lobe of the pituitary and the thyroid or of the sex glands produces the reverse effect as in dystrophia adiposo-genitalis. Prof. Elliot Smith has suggested this as the cause of the delayed union found in the bones of Tutankhamen (Paterson, *loc. cit.*).

Sidhom *et al.* (1931) carried out a valuable investigation in Egyptian boys of known age. Some epiphyseal centres, notably the lower end of the humerus, were found in this series to unite earlier than in Europeans. It will be found by an examination of the tabulated results of this present examination that union in Indians takes place considerably earlier than in any other race of which I can find statistics. Such earlier ossification is after all not surprising in view of the fact that Indians, the girls particularly, undoubtedly reach the age of puberty and mature much earlier than Europeans.

It has been stated by experts on the subject of nutrition, that the average calcium intake of the Indian child is much less than that of the average European. I have tried to obtain figures showing the comparative blood-calcium content in both races. Such figures as are available are vitiated by the fact that they relate mainly to diseased cases and so cannot be accepted as normal. Much work remains to be done in this sphere. The available figures, however, show no great discrepancy in the blood-calcium content of Indian as compared with European children. It is more than possible that the conditions of endocrine balance created by a superabundance of ultra-violet radiation and warmth tend to produce a condition whereby a greater proportion of the calcium intake is assimilated and made available for the formation of osseous tissue.

Many variations from the normal have been met with, such as double epiphyseal centres, the pseudo-epiphyses described by Wakeley (1924), and accessory ossicles and centres. These have been placed in the Appendix.

As stated above, the normal endocrine balance, if upset by pathological states, can produce remarkable variations from the normal rate of formation and union of osseous centres. Conditions such as acromegaly, or dyspituitarism, cretinism and the like, all present variations from normal ossification. One case, of which the photographs and skiagrams were kindly lent to me by Dr. M. Mukherji, has all the appearances, physical and radiological, of a boy of 12 to 14 years. He was 32 years old (Plate X). Another case which I examined at the Medical College Hospitals, aged 20, had the appearance and the radiological characteristics of a boy of 12 (Plate XI). In this case, however, the skiagrams showed definite evidence of some



FIG. 1.



FIG. 3.



FIG. 2.

Figs. 1, 2 and 3.—Case of a Hindu male aged 32. The radiological appearances are those of a boy of 12.



FIG. 5.



FIG. 4.



FIG. 6.

Figs. 4 and 5.—Case of a Hindu male aged 20. Note the sclerosis at the zone of proliferation—probably a case of a foetal rickets.

Fig. 6.—Skiagram showing the mode of fusion of the compound epiphysis for the lower end of the

degree of achondroplasia, with zones of sclerosed bone on each side of the zone of proliferation.

Clark (1936) has used roentgenographic methods in an estimation of metabolic rate in such cases of children presenting signs of endocrine disturbance. His paper is valuable in showing the variations from normal ossification that can take place in such circumstances.

Morrison and Bogan (1927) in a study of sixty-eight diabetic children have shown that the times of ossification were more advanced than normal in pre-diabetic children, and in those whom the onset of the disease was recent. When diabetes had been known to be present for three years or more, ossification was shown to be retarded.

Gray and Geyman (1933) confirmed the findings of Morrison and Bogan.

It is doubtful if the pancreas itself is responsible for these changes. It would seem more probable that an endocrine imbalance created as a result of disease is the more likely cause. The matter is of importance in India where the incidence of diabetes is high. All figures relating to ossification in diabetic subjects must be treated with caution.

In addition to such variations in the rate of ossification caused by undoubted changes in metabolism, there are many pathological conditions which have to be considered in individual cases, such as trauma, infections—septic and syphilitic, rickets—the chondro-dystrophies and osteo-chondritis. This by no means exhausts the list.

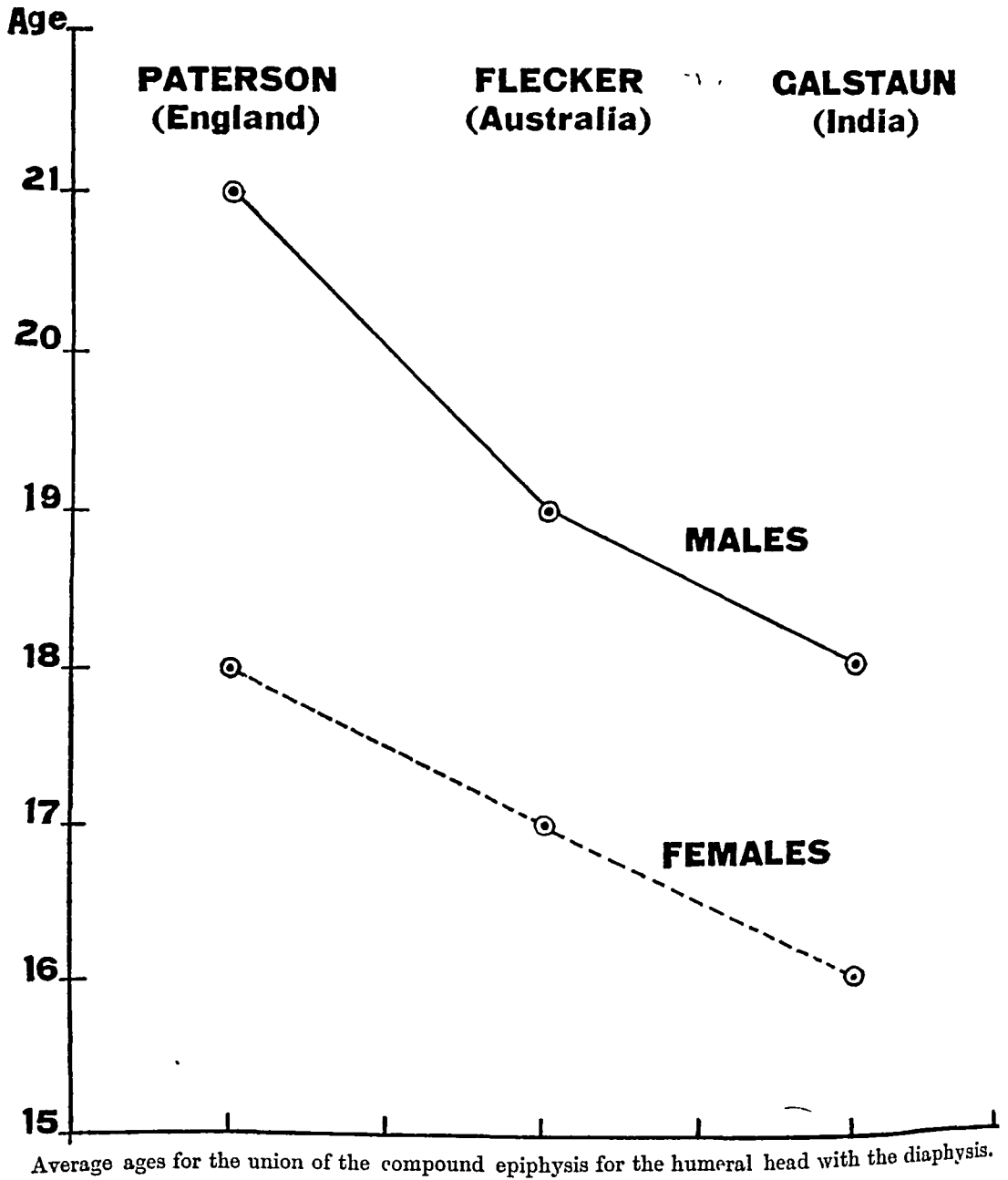
The following series of *x*-ray findings are all in respect of Indian subjects of known age. No distinction has been made of caste or creed. The majority of the cases examined have been either Hindus or Mohammedans of Bengal. No difference of any appreciable extent has been found in the ossification of these two main groups. A great deal more work is however necessary on this subject. It is possible for example that the times of union of epiphyses are not quite the same in a Pathan and a Madrassite. A further investigation on these lines should include all the main types of races found in India, a few cases of each being examined, girls and boys at the various ages, in order to ascertain any differences that might exist.

Pillai (1936) has published a paper dealing with the rate of union, i.e., the time taken from the commencement to the completion of union of epiphyses and diaphyses. Though this thesis does not specifically deal with this aspect of the subject—Pillai's results do not agree with ours in Bengal in this respect. It is our experience here that the whole process of union is complete in less than six months—and less than this in the case of small bones such as metacarpals and phalanges. This is in agreement with Flecker. It is my intention, however, to verify this work with a few selected cases. It must be remembered in this connection that the epiphyseal scar after union has been completed may persist for years.

If an adequate survey were made it would not be surprising to find a gradual acceleration of ossification—as one moved from regions inhabited by races such as the Nordic, to those in the Tropics. Whether, as I have suggested, such acceleration is due to an increased metabolic activity owing to an abundance of

ultra-violet rays and warmth, combined with skin pigmentation enabling certain biochemical changes to take place influencing *pari passu* maturity and ossification, is an interesting speculation.

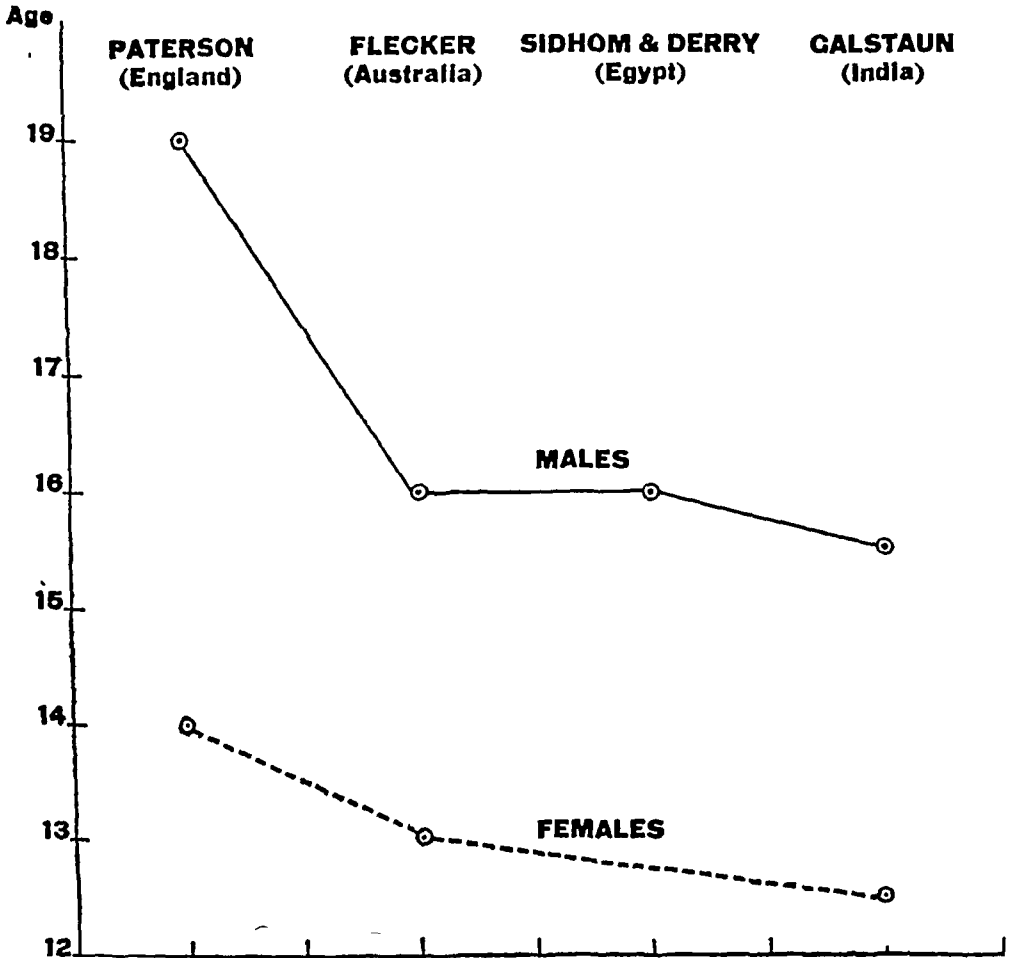
GRAPH 1.



In my previous paper on the union of centres in Indian girls, I attempted to divide the degree of union into four groups, viz., just commencing, commencing, nearly complete, and complete union. This method has been found to be unduly

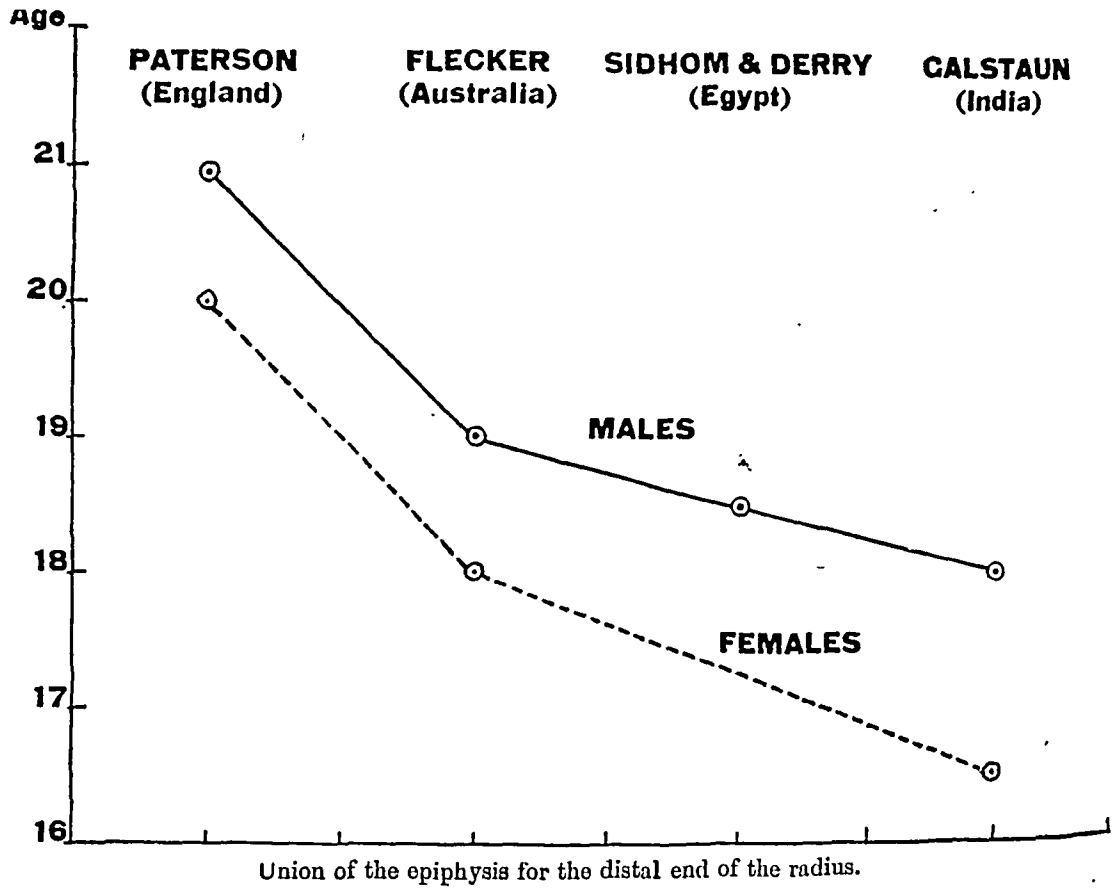
cumbersome and I have discarded it in this paper. Sidhom *et al.* (*loc. cit.*), in realizing the difficulty of determining accurately by skiagrams the amount of union that has taken place between epiphysis and diaphysis, have divided the process into three stages.

GRAPH 2.

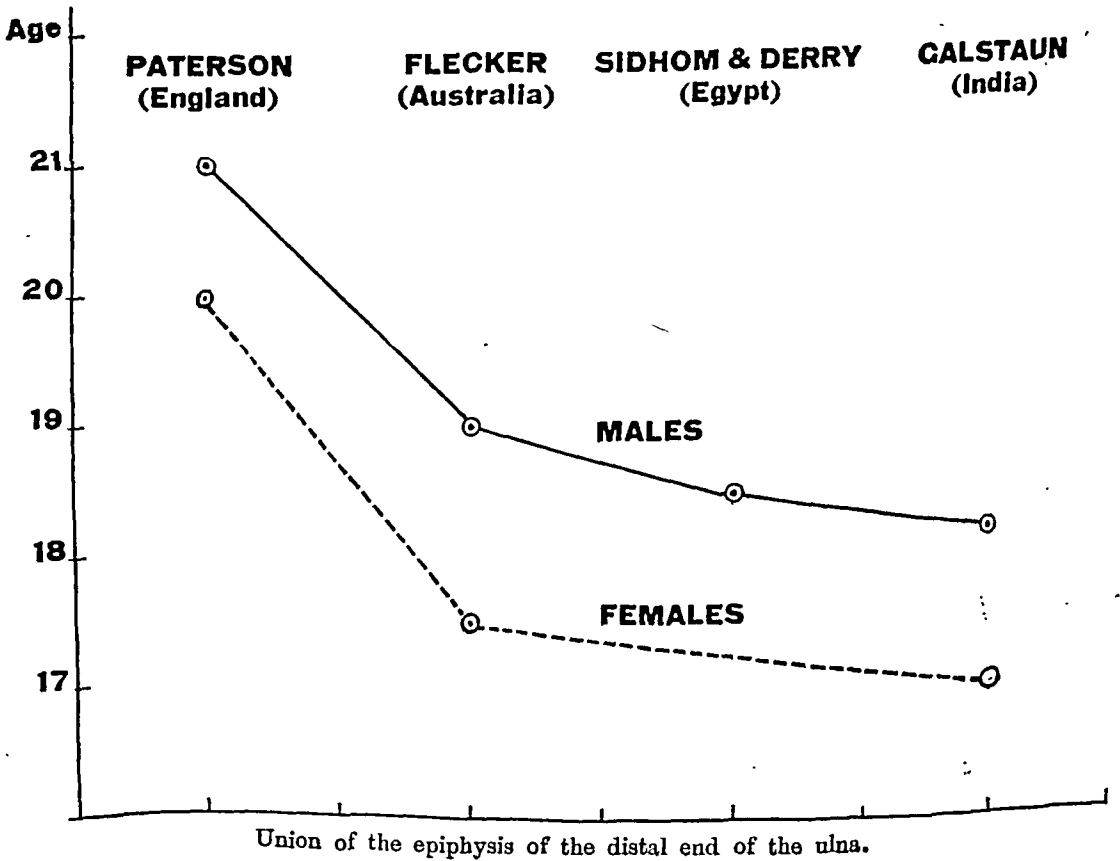


Average ages for the union of the common distal epiphysis of the humerus with the diaphysis.

In the first stage the interval between epiphysis and diaphysis is occupied by cartilage and skiagrams show a very definite gap between the two. A section taken through this growing end of the bone shows that the cartilage is continuous with, or attached to a very thin smooth plate of bone on both the epiphyseal and diaphyseal surfaces. The process of osteogenesis and osteoclasia takes place in association with these thin plates of bone. They are said to be more marked in



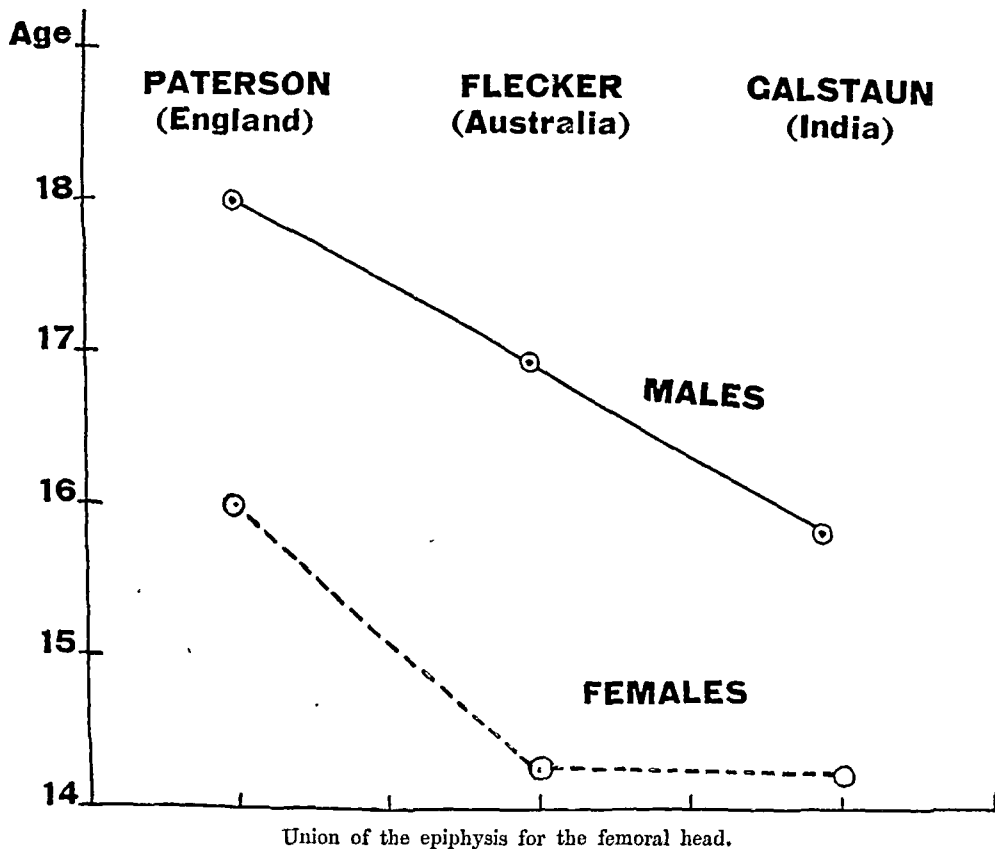
GRAPH 4.



relation to the diaphyseal plate than to the epiphyseal one. Sections show that the cartilage cells arranged in their usual orderly rows increase in size towards the diaphyseal plate, while on the epiphyseal side they are fewer in number and more irregular. It would seem from the above that the process of osteogenesis is more marked on the diaphyseal side, while at the epiphyseal side osteoclasts takes place with the formation of cancellous bone.

In this manner the bone lengthens while the bony plates remain the same thickness. Near the time of union the cartilaginous space becomes less wide and

GRAPH 5.



the two bony plates eventually fuse, with the cessation of growth. This ends the first stage.

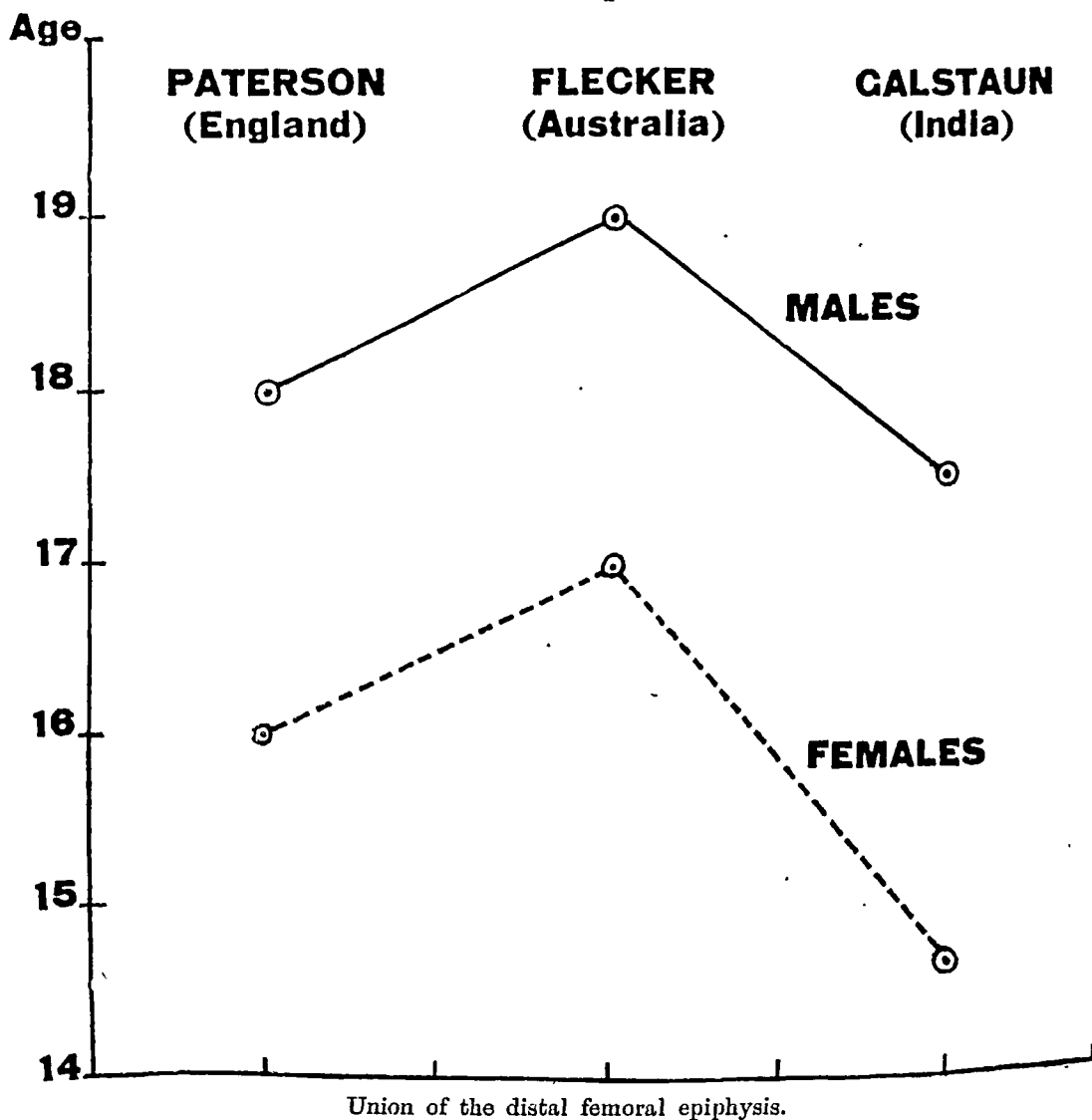
In the second stage the single plate of bone formed by the fusion of the epiphyseal and diaphyseal plates is often seen in skiagrams.

The third stage described by Sidhom *et al.* is reached when this bony plate is entirely absorbed and replaced by spongy bone. As mentioned by the above authors, this process may take months or several years.

This third stage is in our opinion hardly to be regarded as anything but a sequel to the process of union. It is nothing more or less than what has been described as the epiphyseal scar. Were such a bone to be macerated, epiphysis and diaphysis would remain attached.

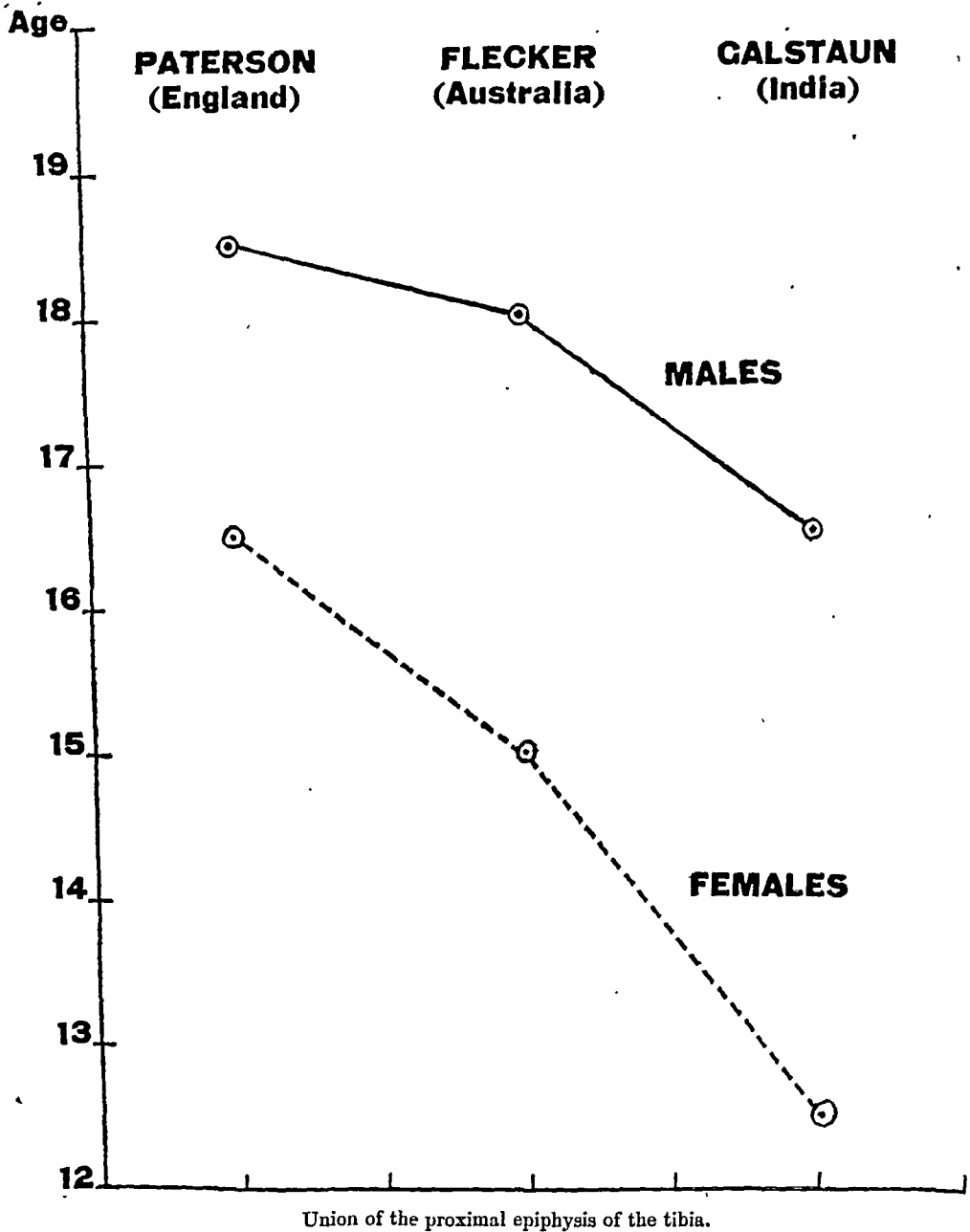
In this paper centres have been taken as united when definite bony architecture is seen between epiphysis and diaphysis, in such a manner that, as

GRAPH 16.



far as one might ascertain by radiological appearances, the centres would remain united had the bone been subjected to the process of maceration. In some cases both sides, e.g., both hands and feet, have been compared. No great disparity has been found in ossification, though slight differences have sometimes been observed. This is in accordance with the findings of Köehler. I have not been

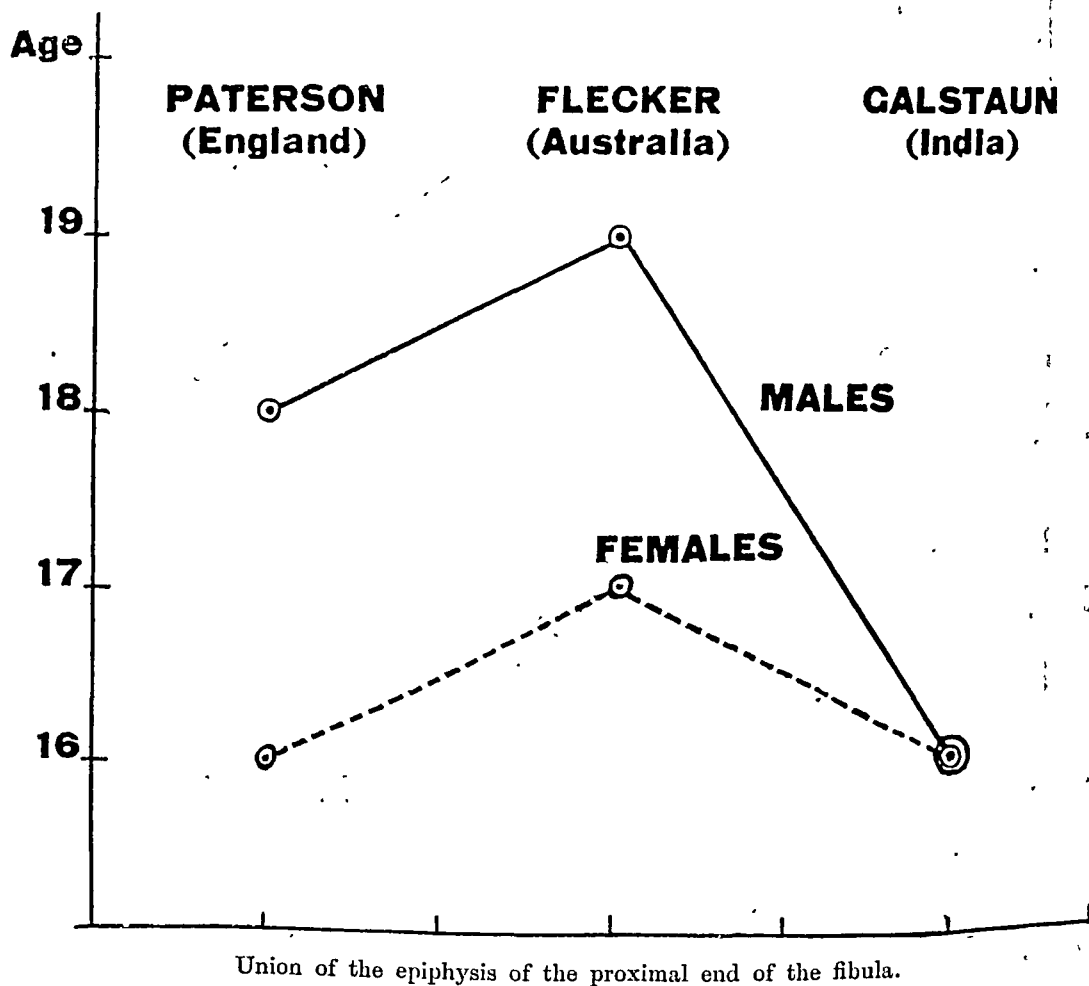
GRAPH 7.



able to confirm Pryor's (*loc. cit.*) findings—he states that ossification is always bilaterally symmetrical. While this has been found generally to be the rule, as stated above slight variations have sometimes been found.

It has been suggested to me that this investigation should include some survey of the possible effects of varying types of nutrition on ossification. Schinz, Baensch and Friedl (1928) have in fact found that variations in ossification and epiphyseal union occur as a result of differences in social position, quoting the

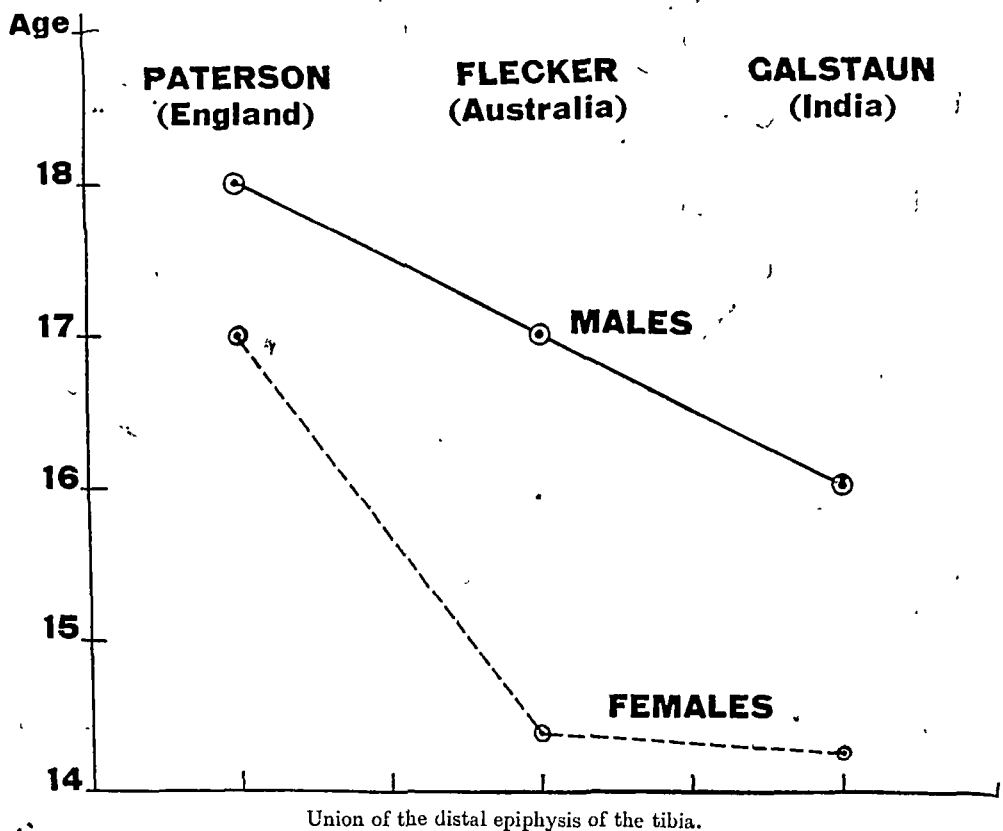
GRAPH 8.



children of peasants, artisans, and well-to-do people. I have not been able to confirm this in India, but further study on the lines of differences found with variations of diet and nutrition is indicated. The relatively high incidence of osteomalacia in women shut up in zenanas without adequate light and fresh air is a pointer to the variations in ossification that might be expected in these subjects.

The present series of cases has been drawn from all strata of the population of Calcutta—from very well-nourished families such as those of some of my medical colleagues, to some poor orphanages, the only qualification being the certainty of age. They do not show any difference in ossification which could be put down to nutrition. It is possible, however, that in cases of tribes or castes which are very definitely under-nourished that differences may exist. Dr. Aykroyd at the Fourteenth Conference of the workers under the Indian Research Fund Association mentioned one tribe in Southern India whose staple diet was tapioca—the dietary

GRAPH 9.



in question containing only half of what is considered to be the minimum permissible phosphate intake. The stature of these people was three inches below normal. In cases such as this it may be found that differences in ossifications exist. More work should be done in this connection.

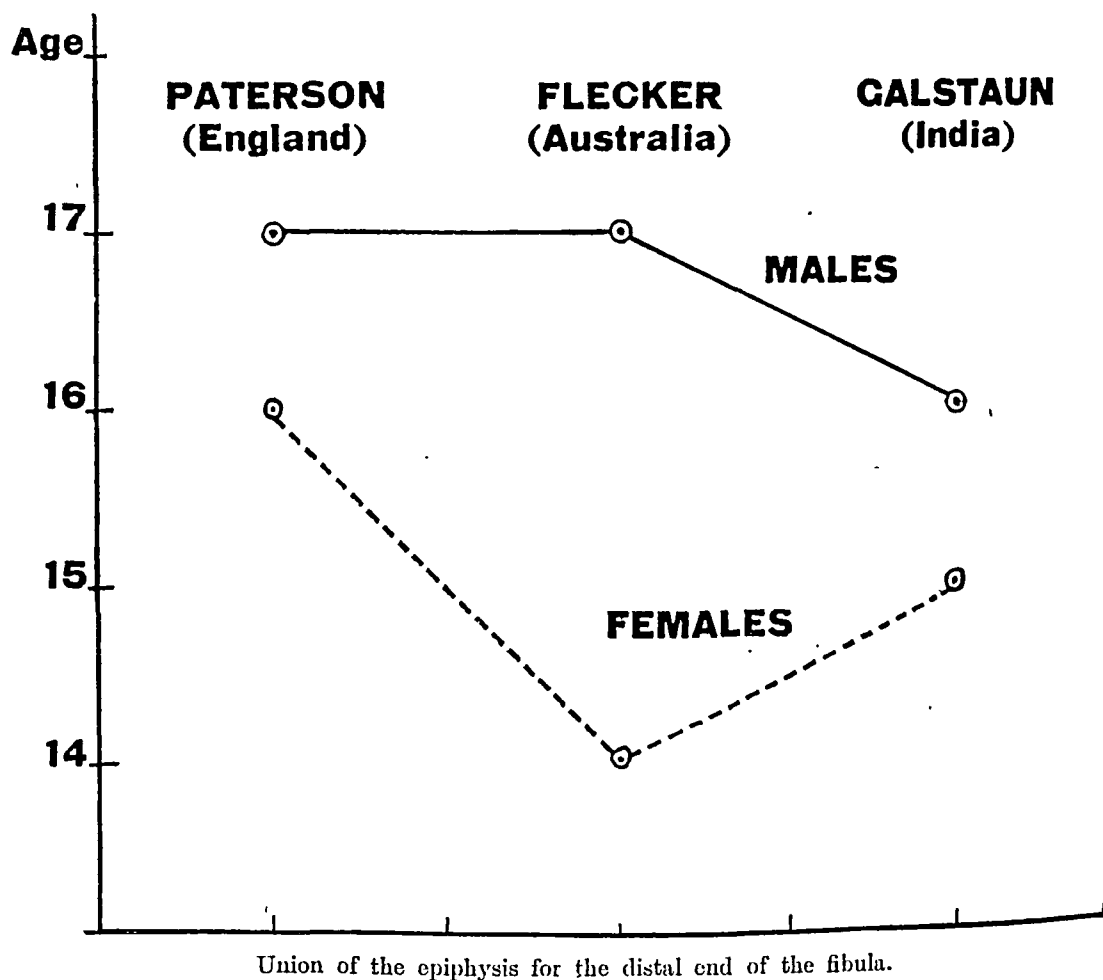
The figures given in the following tables have been worked out

- by an ordinary compilation of my findings,
- by the statistical machine.

In all nearly seven thousand skiagrams have been taken and examined in the course of this work.

The only paper bearing directly on this subject, apart from my own in April 1930, is a very short account by Hepworth (1929). In this communication boys and girls are not separated and so the figures given in this small series are of little value.

GRAPH 10.



CLAVICLE.

The main mass of this bone is said to appear in the fifth or sixth week of foetal life (Cunningham, 1913).

A separate centre exists for the inner end. This was found to appear as a thin bony plate usually at between the ages of 15 and 19 in boys, fusing at round about 22 years. In the case of girls the centre appeared at 14 to 16 years, but fused earlier, 20 being the average age. The difficulty of properly seeing this centre owing to overlapping of bony structures such as vertebræ, ribs, costo-transverse articulations and the like, can often be overcome by slightly inclining

the patient. Our findings show a much earlier appearance of this centre than described by Flecker who gives figures of 21 years for appearance and 22 for fusion, in both sexes. In one male examined, aged 22, the centre was seen to have fused on the left side but not on the right. It had not appeared in one boy aged 20.

TABLE I.

The epiphysis for the medial end of the clavicle.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
12	23	64	0	0
13	22	26	0	0
14	30	26	2	0
15	21	71	1	3
16	37	53	20	3
17	11	37	10	16
18	21	21	21	9	..	0
19	5	19	5	12	..	4
20	15	41	5	20	10	19
21	4	12	..	4	4	8
22	9	29	..	8	9	21
23	4	14	..	3	3	11
24	5	14	..	2	5	12
25	2	19	2	19

SCAPULA.

The main mass of the body of this bone appears in intra-uterine life—at eight weeks or so. It is fairly well formed at birth. The separate centres of ossification for the acromion and coronoid processes will be described under their own names. The centres described by Cunningham for the lower glenoid lip and vertebral border were never seen. The centre for the inferior angle of the scapula was seen in four cases, all round about sixteen years. No evidence could be obtained as to its time of appearance or fusion.

The coracoid.—This component of the scapula has been found to develop from three centres—one for the main mass, one for the tip, and one for the angle of the process. The two latter are not always seen.

The centre for the main mass was seen at birth in two newly-born girls examined and one boy out of three of each.

It was seen in both boys and girls from 2½ months onwards, and appears to unite with the main mass of the scapula at about 2½ years in both sexes. It is a difficult centre to be sure of as there is a good deal of bony overlapping.

The centre for the coracoid tip was seen in one case (male) at 8 years. It appeared more usually at between 10 and 11 years in both sexes with a tendency to appear earlier in girls. It was usually seen to unite round about 16 years.

The epiphysis for the angle of the coracoid process is seen in the form of a thin plate of bone at the junction of the horizontal and ascending rami medially.

It was not constantly seen, but appeared at 8 to 10 years in girls and 10 to 14 in boys. It seems to fuse at 16 in girls and 17 to 18 years in boys.

TABLE II.

Coracoid main mass.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
Under 1 year.	7	4	4	3
1	2	4	2	4
2	5	6	3	1	2	5
3	4	5	1	2	3	3
4	7	5	7	5
5	4	4	4	4
6	1	3	1	3

The acromion.—The base of the acromion is formed from the original main mass of the bone. A separate centre appears for the tip. The times of appearance and fusion of this centre were found to be as follows:—

		Age of appearance.	Age of fusion.
Males	13 to 16	14 to 19
Females	12 to 14	14 to 17

The majority of the male cases appeared at 15 to 16 years and fused at 19 years as compared with 12 to 14 for appearance and 16 for fusion in the case of females.

Cunningham	..	Appearance 16-17	Fuses 20
Flecker	Appearance 14 girls,	15 boys.
		Fuses 16-17 girls, and 17-19 boys.	

TABLE III.

Acromion.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
1
2
3
4
5
6
7
8
9
10

TABLE III—concl'd.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
11
12	21	..	10
13	21	25	13	3
14	28	25	23	7	1	1
15	19	64	14	25	5	..
16	28	50	15	34	13	6
17	1	16	..	9	1	7
18	7	9	1	6	6	3
19	2	6	1	..	1	6
20	..	29	29
21	..	8	8
22	..	3	3

THE HUMERUS.

The centre for the shaft of this bone appears at about the eighth week of intra-uterine life (Cunningham, *loc. cit.*). The centres for the epiphyses and lower end have been studied.

The head of the humerus.—The upper end of the shaft of the humerus at birth is shaped like a sharp wedge. On the medial aspect of this wedge appears the centre for the head of the humerus and on the lateral aspect that of the greater tubercle. The lesser tubercle appears in the interval between the two, after they have fused.

The main centre for the head was found to appear during the first year of life in both boys and girls. It was not seen in the newly-born cases examined. This is in keeping with both Cunningham (*loc. cit.*) and Flecker (*loc. cit.*). Davies and Parsons (1927) quote having found it present in two out of four cases at birth. It was seen in our series in a boy aged 1 month and two girls aged 2½ months.

The greater tubercle of the humerus.—A separate centre of ossification definitely appears for the greater tubercle and so far as we have been able to ascertain also for the lesser. Cohn (1924) is doubtful even about the former. Before this investigation, however, it was described among others by Borovansky and Hnevkovsky (1929) and Flecker (*loc. cit.*). Cunningham (*loc. cit.*) quotes it as a separate centre appearing at from two to three years of age. This is in agreement with Paterson's (*loc. cit.*) figures. Flecker has described it as appearing at 9 and 11 months. I have seen it at 7 months in a girl and 10 in a boy. It appears to fuse with the centre for the lesser tuberosity at between 5 and 9 years in both boys and girls and with the centre for the head at between 2 and 4 years.

The lesser tubercle of the humerus appeared in one series at between 5 and 7 years. It fuses with the greater tuberosity in a very short time after its appearance. It is a difficult centre to demonstrate clearly in a skiagram. It is necessary to carefully rotate the arm to see it.

Fusion of the main mass of the humeral head with the shaft.—These centres were found to unite at between 14 and 18 in boys, and 14 and 16 in girls. These

figures are considerably earlier than those given by Cunningham (*loc. cit.*) who gives this age at 25 years, and Flecker (*loc. cit.*) who gives it as between 16 and 20. Tables IV to IX show the comparison of figures.

TABLE IV.

The appearance of the epiphysis for the head of humerus, and union of the compound epiphysis with the shaft.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
At birth.	3	3
Under 1 year.	8	6	7	5
1	3	6	3	6
2	9	10	9	10
3	6	7	6	7
4	8	10	8	10
5	8	5	8	5
6	7	10	7	10
7	9	20	9	20
8	10	33	10	33
9	11	16	11	16
10	22	43	22	43
11	4	44	4	44
12	21	64	21	64
13	21	25	21	25
14	28	25	27	24	1	1
15	19	64	10	63	9	5
16	28	50	23	45	5	7
17	1	16	..	9	1	7
18	7	9	..	2	7	6
19	2	6	2	

TABLE V.

The greater tubercle of the humerus.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
Under 1 year.	8	6	3	2
1	3	6	3	4
2	9	10	8	10
3	6	7	6	6
4	8	10	8	10
5	8	5	8	5
6	7	10	7	10
7	9	20	9	20
8	10	33	10	33

TABLE VI.

Fusion of head of humerus with greater tubercle.

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
2	9	10	1	1
3	6	7	3	3
4	8	10	8	8
5	8	5	7	5
6	7	10	7	10
7	9	20	9	20
8	10	33	10	33

TABLE VII.

The lesser tubercle of the humerus.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
2	9	10	1	..
3	6	7
4	8	10	0	..
5	8	5	5	..
6	7	10	5	7
7	9	20	9	20
8	10	33	10	33

TABLE VIII.

Fusion of head of humerus with greater and lesser tubercles.

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
5	8	5
6	7	10	5	5
7	9	20	7	15
8	10	33	9	32
9	11	16	11	16
10	22	43	22	43

TABLE IX.¹*Fusion of the head and greater tubercle and lesser tubercle with the shaft.*

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
14	28	26	1	1
15	19	65	9	1
16	28	59	5	5
17	1	16	1	7
18	7	9	7	7
19	2	6	2	6

The lower end of the humerus.—The lower end of the humerus is developed from two main epiphyseal centres. The first of these is a compound centre formed from three elements, the centres for the capitellum, trochlea, and lateral epicondyle which eventually fuse to form a single mass. The centre is a separate one from which the medial epicondyle originates and which unites with the inner part of the lower end of the shaft quite separately.

The compound centre for the capitellum, trochlea, and lateral epicondyle.—This centre merits description before the actual dates of ossification of this region are analysed.

Capitellum.—The capitellum is the first to appear here. It was seen in girls at 7 and 10½ months and a boy at 5 months. It was usually present in both sexes at three years. The centre rapidly assumes a well-defined shape with clear-cut outlines.

Trochlea.—The trochlear centre usually appears in girls at 10 years and in boys at 11 years. It was seen, however, in one girl at 7 years and two boys at 9.

More often than not it has an irregular outline—it is often fragmented. It is difficult to be sure of the earliest appearance of the trochlea owing to the overlapping of the bony mass of the olecranon.

Fusion of the centres for the capitellum and trochlea are not quite regular. The relatively late appearance of the trochlear centre is often followed very shortly after by its fusion with the capitellum, and often of the joint centre with the shaft. For this reason it is difficult to estimate the time of union with any accuracy.

The average age for the union of capitellum and trochlea appears to vary from 9 to 13 years in girls and 11 to 15 years in boys.

Lateral epicondyle.—The centre for the lateral epicondyle, like that of the trochlea, both appears and fuses somewhat irregularly. It is often fragmented. The average age for the appearance of this centre in girls was 10 years and in boys 12 years.

Fusion with the capitellum usually took place at between 10 and 12 years in girls and between 11 and 16 years in boys. Sometimes it was found that the lateral epicondyle had fused before the trochlea had done so, or had even appeared in some cases.

In some 30 per cent of cases no separate centre seemed to appear, the process fashioning itself from a bud-like extension of the capitellum, which latter becomes a tongue-like process pointing upwards at the outer side of the diaphysis. Contrary to Flecker, I find that Paterson (*loc. cit.*) was correct in this matter. Flecker, in attempting to discount Paterson's findings, has said that intermediate stages in this tongue formation are not seen. We have very definitely seen many such stages, varying from a small lip to a fully formed tongue.

Many variations are seen in the ossification of this region, e.g., in one boy of 12 the capitellum had appeared and fused with the shaft and lateral epicondyle, though the latter had not completely united with the shaft and was present as a tongue. The trochlea in this case had not yet fused with the capitellum or shaft.

In some cases the trochlea was seen to have appeared and fused with the capitellum, while the centre for the lateral epicondyle had not yet appeared. Sometimes the centre for the lateral epicondyle appeared before that of the trochlea. In one case, a girl aged 9, the capitellum had united with the trochlea but not with the lateral epicondyle which had already appeared at this early age.

Below appears an analysis of our findings in respect of this epiphysis (Tables X to XV):—

TABLE X.

Capitellum.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
At birth.	3	3
Under 1 year.	6	7	3	3
1	4	6	4	5
2	8	6	6	6
3	4	3	4	3
4	4	9	4	9
5	12	5	12	5
6	6	8	6	8
7	8	21	8	21
8	11	32	11	32
9	11	16	11	16
10	17	42	17	42
11	4	44	4	44
12	23	64	7	57	16	7
13	27	26	6	20	21	6
14	51	27	..	17	51	10
15	35	64	..	33	35	31
16	41	50	..	8	41	42
17	17	16	..	1	17	15
18	16	9	16	9

TABLE XI.
The lateral epicondyle.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
9	11	16	2
10	17	42	8	1
11	4	44	1	1
12	23	64	9	30	12	2
13	27	26	13	11	14	4
14	51	27	2	14	49	4
15	35	64	1	9	34	26
16	41	50	..	13	41	37
17	17	16	..	2	17	14
18	16	9	16	9

TABLE XII.
Trochlea.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
7	8	21	1
8	11	32	2
9	11	16	5	2
10	17	42	8	6
11	4	44	2	16
12	23	64	8	34	15	6
13	27	26	10	16	17	5
14	51	27	1	17	40	7
15	35	64	..	33	35	30
16	41	50	..	12	41	38
17	17	16	..	1	17	15
18	16	9	16	9

TABLE XIII.
Union of capitellum and trochlea.

Age.	Number of girls examined.	Number of boys examined.	Union in girls.	Union in boys.
8	11	32	1	..
9	11	16	1	..
10	17	42
11	4	44	..	4
12	21	64	2	9
13	20	26	1	6
14	27	27	..	2
15	19	64	..	5
16	28	50

TABLE XIV.

Union of capitellum and lateral epicondyle.

Age.	Number of girls examined.	Number of boys examined.	Union in girls.	Union in boys.
9	11	16
10	17	42	4	..
11	4	44	..	2
12	21	64	3	3
13	20	26	1	1
14	27	27	..	2
15	19	64	..	2
16	28	50	..	4

TABLE XV.

Union of capitellum, trochlea, and lateral epicondyle.

Age.	Number of girls examined.	Number of boys examined.	Union in girls.	Union in boys.
9	11	16
10	17	42	2	..
11	4	44	1	..
12	21	64	4	8
13	20	26	5	5
14	27	27	3	13
15	19	64	..	21
16	28	50	..	9

UNION OF THE DISTAL HUMERAL EPIPHYSIS WITH THE SHAFT.

The manner of the union of the compound centre above described with the shaft is not constant. Sometimes the three components fuse together, the mass so formed uniting with the shaft, while in other cases the capitellum fuses first and the others follow suit, having occasionally not even appeared before this fusion of the capitellum has taken place. More often the trochlea and lateral epicondyle are present as tongue-like extensions on either side of the capitellum, the space between tongue and shaft below in the case of the trochlea, and laterally in the case of the lateral epicondyle eventually filling up with osseous tissue.

THE CENTRE FOR THE MEDIAL EPICONDYLE.

This centre is next in order of appearance to the capitellum. Like the latter it is usually well formed and does not present the irregularities in shape and

ossification that characterize the trochlea or lateral epicondyle. The epiphysis for the medial epicondyle was found to appear most commonly at 5 years in girls and 7 years in boys, uniting with the shaft at 14 and 16 years respectively.

TABLE XVI.

The medial epicondyle.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
3	4	3
4	4	9	3
5	12	5	7
6	6	8	4	2
7	8	21	8	5
8	11	32	11	12
9	11	16	11	12
10	17	42	17	34
11	4	44	4	41
12	23	64	19	61	4	3
13	27	26	19	26	8	..
14	51	27	12	25	39	2
15	35	64	..	58	35	6
16	41	50	1	32	40	18
17	17	16	..	7	17	9
18	16	9	..	1	16	8
19	5	6	5	6

THE RADIUS.

Ossification commences in the shaft of the radius during the fifth week of foetal life. In addition to this diaphyseal centre, there are separate epiphyses for the head and distal end.

The epiphysis for the head of the radius.—The epiphysis for the head of the radius was found to have appeared in two boys out of nine at the age of four. It has appeared in one girl out of four at the age of four. The majority ages for the appearance of this centre was found to be six in the case of girls and eight in the case of boys. These figures are rather later than those given by Paterson, who found that the centre had appeared in girls during the fifth year and was invariably present in the sixth year and in the case of boys appeared during the sixth and seventh years. Flecker similarly reports a rather earlier appearance. His figures were on the whole similar to those of Paterson.

Fusion with the diaphysis was found to have taken place in six out of twenty-three girls examined and one out of sixty-four boys at the age of 12. The majority ages for boys and girls were found to be 14 and 16 respectively. Only one girl out of thirty-five examined had not united at the age of 15; all had united at the

age of 16. In the case of boys the majority age for union of this epiphysis was found to be 16 years. Ten out of sixteen had united at the age of 17 and all had united at the age of 18.

TABLE XVII.

Head of radius.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
3	4	3
4	4	9	1	2
5	12	6	5	2
6	6	8	5	3
7	8	23	8	10
8	11	32	11	28
9	11	16	11	14
10	17	42	17	40
11	4	44	4	43
12	23	64	17	63	6	1
13	27	26	19	26	8	..
14	51	27	7	25	44	2
15	35	64	1	52	34	12
16	41	50	..	21	41	29
17	17	16	..	6	17	10
18	16	9	16	9
19	5	6	5	6
20

The distal epiphysis of the radius.—The distal epiphysis of the radius was found to appear round about the first year in both boys and girls. Fusion with the diaphysis was noted in one out of twenty-three girls at the age of 12, the majority age being between 16 and 17. Only one case out of sixteen was found to be ununited at the age of 18, and from 19 onwards all cases were found to have united.

In boys, fusion was first observed in two out of twenty-six cases at the age of 14, the majority age being 18. Beyond this age, every case was found to have united.

Below will be found a comparison with the findings of other authors:—

Union of the distal epiphysis of the radius.

				Girls.	Boys.
Paterson	20	21
Flecker	18	19
Sidhom <i>et al.</i>	18½
Galstaun	16½	18

TABLE XVIII.

The distal epiphysis of the radius.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3
Under 1 year.	5	4	2
1	3	4	3	2
2	8	7	7	7
3	4	5	4	5
4	4	7	4	7
5	6	5	6	5
6	6	6	6	6
7	7	24	7	24
8	10	32	10	32
9	10	15	10	15
10	18	43	18	43
11	4	44	4	44
12	23	67	22	67	1	..
13	27	25	26	25	1	..
14	51	26	29	24	22	2
15	35	64	16	62	19	2
16	41	50	15	36	26	14
17	17	17	3	9	14	8
18	16	9	1	1	15	8
19	5	6	5	6
20

THE ULNA.

The centre of ossification for the shaft appears early during the second month of foetal life. Centres also appear for the tip of the olecranon process and distal end of the bone.

The epiphysis for the tip of the olecranon.—By far the greater portion of the olecranon is formed as an extension of the shaft of the ulna. A separate epiphyseal centre appears for the tip. The earliest appearance of this centre in our series was in two out of eight girls examined aged seven. It was seen in one out of thirty-two boys at the age of eight. The average time for the appearance of this centre would appear to be between nine and 12 in girls and between 11 and 13 in boys. It will be seen from the accompanying table that the appearance of this epiphysis is not very constant. Fusion was found to take place between the ages of 12 and 17 in boys, the majority at 17 and between 12 and 16 in girls, the majority at 15.

It has been observed in quite a number of cases in our series that as for the lateral epicondyle of the humerus no separate centre seems to exist for the tip of the olecranon, the whole of this process in these cases appearing to be formed from the shaft. It was not possible accurately to estimate the percentage of such cases, as the times for the appearance and fusion of the centre sometimes overlap. My

reason for thinking that no separate centre exists in some of these cases is that, where such centre has existed and united with the shaft, it is usually possible to see some signs of an epiphyseal scar for at least some months after such union has taken place. No such scar existed in these cases, the bone presenting a uniform architecture.

TABLE XIX.

The epiphysis for the tip of the olecranon.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
6	6	8	0	0
7	8	22	2	0
8	11	32	4	1
9	11	16	7
10	17	42	12	6
11	4	44	2	18
12	23	64	12	39	7	3
13	27	26	16	24	11	2
14	51	27	4	23	47	4
15	35	64	2	43	33	21
16	41	50	..	14	41	36
17	17	16	..	4	17	12
18	16	9	16	9
19	5	6	5	6

THE DISTAL EPIPHYSIS OF THE ULNA.

This centre appeared in our series in three girls out of four aged four and one boy out of six at the age of six years. The majority ages for its appearance appear to be between eight and 10 for girls and 10 and 11 for boys. Fusion with the shaft was seen in eight out of fifty-one girls at the age of 14 as against one out of twenty-five boys, the majority ages for girls and boys respectively being 17 and 18 years.

TABLE XX.

The distal epiphysis of the ulna.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
4	4	7	3
5	6	5	1
6	6	6	2	1
7	7	24	6	5
8	10	32	8	10
9	10	15	9	8
10	18	43	16	34

TABLE XX—concl'd.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
11	4	44	4	40
12	23	67	23	66
13	27	26	27	26
14	51	26	43	25	8	1
15	35	64	27	63	8	1
16	41	50	21	46	20	4
17	17	17	3	15	14	2
18	16	9	1	4	15	5
19	5	6	5	6
20	..	29	29

THE STYLOID PROCESS OF THE ULNA.

A separate centre for the ulnar styloid process was seen in three cases in this series. It appeared very shortly after the lower epiphysis had been well formed. This centre is a matter of some importance in view of the fact that this process is more often than not fractured in Colles' fracture.

Comparison of figures for the union of the distal epiphysis of the ulna.

				Girls.	Boys.
Paterson	20	21
Flecker	17½	19
Sidhom <i>et al.</i>	18½
Galstaun	17	18½

THE CARPAL BONES.

The carpal bones, as a rule, all develop from a single centre. Double centres have been described by Wollard for the scaphoid, Pfitzner for the cuneiform, Quain and Flecker for the lunate, Pryor and Borovansky and Hnevkovsky for the trapezoid. We have in our series met one case with a double centre for the scaphoid. Two cases were doubtful in that where double centres were seen in each case which were thought to be the centres for the trapezium, it was impossible to be sure that the second one in each case was not for the trapezoid. The order of appearance of the carpal bones is as follows: first the os magnum which is very shortly followed by the unciform. Then follow the semilunar, trapezium and trapezoid, scaphoid, and last of all the pisiform.

THE OS MAGNUM OR CAPITATE BONE.

Of the carpal bones the os magnum or capitate is the first to appear. It was seen at birth in two out of three girls and one out of three boys. It usually makes its appearance some time during the first year and seems to present itself rather earlier in girls.

TABLE XXI.

The os magnum or capitate bone.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3	2	1
Under 1 year.	4	4	3	2
1	3	4	3	4
2	9	7	9	7
3	4	4	4	4
4	4	7	4	7
5	6	6	6	6
6	6	6	6	6
7	7	24	7	24
8	10	32	10	32

THE UNCIFORM OR HAMATE BONE.

A centre of ossification for the unciform bone was seen in one out of three cases in both boys and girls at birth. The bone is thus next in order of appearance to the os magnum and appears very shortly after it. The average age of appearance is from 8 to 14 months in both sexes.

TABLE XXII.

The unciform or hamate bone.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.
At birth.	3	3	1	1
Under 1 year.	4	4	3	1
1	3	4	3	4
2	9	7	9	7
3	4	4	4	4
4	4	7	4	7
5	6	6	6	6
6	6	6	6	6
7	7	24	7	23
8	10	32	10	32

THE CUNEIFORM BONE OR OS TRIQUETRUM.

This was seen in one out of nine girls and three out of seven boys at the age of 2. The average age of appearance was 2 to 3 for girls and 3 to 4 for boys.

TABLE XXIII.

The cuneiform bone or os triquetrum.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
1	3	4
2	9	7	1	3
3	4	4	4	1
4	4	7	4	7
5	6	6	6	6
6	6	6	5	3
7	7	24	7	23
8	10	32	10	32
9	10	15	10	15
10	18	..	18	..

THE SEMILUNAR OR LUNATE BONE.

This was seen to be present in two out of seven boys aged 2. The average age of appearance is 5 in the case of both girls and boys.

TABLE XXIV.

The semilunar or lunate bone.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
2	9	7	..	2
3	4	4
4	4	7	2	2
5	6	6	4	6
6	6	6	5	1
7	7	24	7	23
8	10	32	10	30
9	10	15	10	15
10	18	41	18	41

THE TRAPEZIUM OR OS MULTANGULUM MAJUS.

Ossification was observed in the case of trapezium in two out of four girls aged 4, and twenty out of twenty-four boys aged 7. In all girls aged 7 the centre was seen and in all boys aged 11. The average age of appearance would appear to be 5 to 7 in the case of girls and from 7 to 11 in the case of boys.

TABLE XXV.

Trapezium or os multangulum majus.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
1	3	4
2	9	7
3	4	4
4	4	7	2	1
5	6	6	3	1
6	6	6	4	0
7	7	24	7	20
8	10	32	10	17
9	10	15	10	13
10	18	41	18	39
11	4	44	4	44

THE TRAPEZOID BONE OR OS MULTANGULUM MINUS.

The centre for this bone was seen in one out of six cases examined in boys aged 5. Nineteen out of twenty-four boys had it at the age of 7. In the case of girls the centre appears rather earlier, being seen in two out of four girls at the age of 4 and all cases at the age of 7 or more.

TABLE XXVI.

The trapezoid bone or os multangulum minus.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
1	3	4
2	9	7
3	4	4
4	4	7	2	0
5	6	6	3	1
6	6	6	5	1
7	7	24	7	19
8	10	32	10	22
9	10	15	10	14
10	18	41	18	41

THE SCAPHOID OR NAVICULAR BONE.

Ossification was seen in the scaphoid in one out of four girls aged 4, two out of six aged 5, and constantly from the age of 7 onwards. In the case of boys it was seen in ten out of twenty-four aged 7, thirty-nine out of forty-one aged 10, and constantly from the age of 11 upwards.

TABLE XXVII.

The scaphoid or navicular bone.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
4	4	7	1	..
5	6	5	2	..
6	6	6	5	..
7	7	24	7	10
8	10	32	10	20
9	10	15	10	12
10	18	41	18	39
11	4	44	4	44

THE PISIFORM BONE.

Ossification was observed in the pisiform bone at a much later date than in the carpal bone. In girls it was seen in one case out of ten aged 8 and constantly from the age of 12 upwards. In boys it was first seen at the age of 11 in six out of forty-four cases. It was present in twenty-two out of twenty-six aged 14, fifty-nine out of sixty-four aged 15, forty-nine out of fifty aged 16, and constantly from the age of 17 upwards. These figures appear to be considerably later than those given by Flecker or Paterson.

Table XXIX shows a comparison of the average dates of appearance of the carpal bones as quoted by Cunningham, Paterson, Flecker and our series.

Overlapping of the newly-appeared pisiform with the triquetrum was a source of error that was not overlooked and every case was carefully examined with this possibility in view.

TABLE XXVIII.

The pisiform bone.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
8	10	32	1	..
9	10	15	3	..
10	18	41	8	..
11	4	44	1	6
12	21	67	21	34
13	20	26	20	16
14	27	26	27	22
15	19	64	19	59
16	28	50	28	49
17	1	16	1	16

TABLE XXIX.

A comparison of the times of appearance of the carpal bones.

	CUNNINGHAM.	PATERSON.		FLECKER.		GALSTAUN.	
	Both sexes.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.
Capitate .	11/12 months	Under 6 months.	6 months	6 months	6 months	6 months	6 months
Hamate ..	12/14 months	Under 6 months.	6 months	10/11 months.	10/11 months.	8/14 months.	5/14 months.
Triquetrum	3	2 to 3	3 to 4	3 to 4	4	2 to 3	3 to 4
Lunate ..	5 to 6	3 to 4	4 to 5	3	4	5	5
Multangulum majus.	6	4 to 5	6	5	5	5 to 6	7
Multangulum minus.	6 to 7	4 to 5	6	6	6	5 to 6	4 to 7
Scaphoid ..	6	6	6	4	6	6	7 to 11
Pisiform ..	10 to 12	9 to 10	12 to 14	10	11	9 to 12	12 to 17

THE METACARPALS.

Like other long bones the metacarpals are developed from a primary centre for the shaft, but unlike the majority, usually only have one epiphyseal centre. The primary centre in the case of the second, third, fourth, and fifth metacarpals appears at about the ninth or the tenth week of fetal life. The centre for the first metacarpal appears a little later. The epiphyseal centres for the second, third, fourth, and fifth metacarpals, like those of the phalanges, appear at the base in contra-distinction to the others where the centre appears at the head. If it is borne in mind that the first metacarpal and metatarsal morphologically are phalanges, this is to be expected. Cunningham quotes Broom (*Anat. Anz.*, 28) who by a comparison to reptilian types explains this difference in ossification, in that, the ends of the bones that enter into formation of joints possessing the freest movement are the ends where the epiphyses usually appear.

The first metacarpal.—The shaft and head of the first metacarpal are formed from the primary ossific centre as in the phalanges. The base is developed from an epiphysis.

In 5 per cent of cases examined, as shown by the accompanying table, a second epiphyseal centre was found for the head. Borovansky and Hnevkovsky found this epiphysis in 50 per cent of Prague boys and suggest that owing to a fissure often seen the percentage might actually be 70. A careful scrutiny of our cases does not confirm this. Paterson suggests that the second epiphysis is hereditary and quotes a case in which it was seen in a father and two sons. Wakeley has described these centres as pseudo-epiphyses and has recorded some unusual ones including bilateral centres in the base of the second metacarpal.

Our series showed that one out of eight girls and two out of seven boys had the epiphyseal centre for the first metacarpal at the age of 2. All of three girls aged 3 showed it. It was present in all boys and girls from the age of 5 onwards.

Union of the epiphysis for the shaft was present in the majority of girls aged 14 and all aged 17. In the case of boys the majority age was found to be between 16 and 17. All cases aged 18 or more were found to have united.

Second, third, fourth, and fifth metacarpals.—As described above, the epiphyseal centre for this bone is at the head, though additional centres have occasionally been described by both Wakeley and Paterson as occurring at the base. The epiphyseal centres for the second, third, fourth, and fifth metacarpals appear rather earlier than that of the first metacarpal. It was seen in one girl aged 7 months. More than half the cases were ossified at the age of 2. At the age of 3, all the girls were found to have the centre. Three out of four boys showed appearance.

Fusion in these epiphyses, in the majority of cases, takes place at the age of 14 in girls and 15 in boys.

TABLE XXX.

First metacarpal.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
Under 1 year.	7	6	0	0
1	3	4	0	0
2	8	7	1	2
3	3	4	3	0
4	4	7	4	5
5	6	6	6	5
6	6	6	6	5

TABLE XXX—concl'd.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
13	27	25	6	1
14	51	26	39	2
15	35	64	30	9
16	41	50	37	26
17	17	17	17	11
18	16	9	14	9
19	5	6	5	6

TABLE XXXI.

Second, third, fourth, and fifth metacarpal.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
Under 1 year.	7	6	1	0
1	3	4	1*
2	8	7	4	4*
3	3	4	3	3
4	4	7	4	6
5	6	6	6	6
6	6	6	6	6
13	27	25	5	..
14	51	25	38	2
15	35	64	33	10
16	41	50	40	26
17	17	16	17	11
18	16	9	16	9

* Except little finger.

TABLE XXXII.

A comparison of the times of appearance and fusion of the epiphyses for the metacarpals.

		CUNNINGHAM.		PATERSON.		FLECKER.		SIDHOM <i>et al.</i>		GALSTAUN.	
		Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.
1st metacarpal	Appearance	3	3	3	2	2	Under 2	4	3
	Fusion ..	20	20	19	17	18	16	17/18	17/18	16/18	14/16
2nd, 3rd, 4th, and 5th metacarpals.	Appearance	3	3	3	2	$\frac{3}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{2}$
	Fusion ..	20	20	19	17	18	16	17/18	..	16/18	14/15

TABLE XXXIII.

Instances of the distal epiphysis for the first metacarpal.

Age.	Male.	Female.
9	2	0
10	5	2
11	6	0
12	3	2
13	2	0
14	2	0
15	4	0
16	2	0

Total 30 out of 600 cases examined.

THE PHALANGES OF THE HAND.

Ossification in the shafts of the phalanges commences at about the ninth week of intra-uterine life. An epiphyseal centre appears for the bases of all the phalanges. Table XXXIV will show that there is a good deal of divergence of opinion as to the times of appearance and fusion of these epiphyses. Whereas Flecker has found that the proximal row appears earlier than the others, Paterson found that there was not much difference. Our series in common with that of Flecker showed that the epiphysis for the proximal phalanx for the thumb tended to appear rather earlier than its fellows in the same row.

The appearance of the epiphyseal centres in the proximal row of phalanges was found to be at $1\frac{1}{2}$ years on the average in females and from 2 to 4 years in males. The epiphyses for the proximal phalanx for the thumb, as stated above, usually appear a little later in both sexes. Union with the shaft was found to take place at 14 to 15 years in females and 17 to 18 years in males.

In the middle row of phalanges the epiphyseal centre was seen to appear at from 2 to 4 years in females and at an average of 3 years in males. The little finger was sometimes behind in the appearance of this centre. Union with the shaft was found to take place at 14 to 16 years in females and 16 to 18 in males.

In the terminal row of phalanges appearance of the epiphyseal centre was seen at 3 years in females and 3 to 5 years in males. This latter figure is rather later than that quoted by other authorities. Union with the shaft was found to take place at the age of 15 in females and at 17 to 18 in males.

It is absolutely essential in taking skiagrams of the hand or foot for the purpose of seeing the epiphyseal centres that part to be radiographed be kept absolutely flat. The slightest flexion will obscure an epiphysis by overlapping. Tables XXXIV and XXXV show a comparison of figures given by various authors.

TABLE XXXIV.

The phalanges of the hand.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
<i>Proximal.</i>				
Under 1 year:	6	6	1	0
1	3	4	2†	2†
2	8	7	4*	2 All
			3 All	4*
3	3	3	3	2
4	4	7	4	6
5	6	5	6	5
6	6	6	6	6
<i>Middle.</i>				
1	3	4	0	0
2	8	7	3	1
3	3	3	3	2
4	4	7	4	6‡
5	6	5	6	5
6	6	6	6	6
<i>Terminal.</i>				
1	3	4	0	0
2	8	7	3	0
3	3	3	3§	2
4	4	7	4	4
5	6	5	6	5
6	6	6	6	6

* Except thumb.

† Except thumb and little finger.

‡ Except little finger in 3 cases.

§ Except little finger.

TABLE XXXV.

The phalanges of the hand.

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
<i>Proximal.</i>				
12	23	66	1	..
13	27	25	1	..
14	51	25	36	2
15	35	64	31	10
16	41	50	41	25
17	17	17	17	11
18	16	9	16	9
19	5	6	5	6

TABLE XXXV—concl'd.

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
<i>Middle.</i>				
12	23	66	2	..
13	27	25	1	1
14	51	25	36	2
15	35	64	31	8
16	41	50	41	21
17	17	17	17	10
18	16	9	16	8
19	5	6	5	6
<i>Terminal.</i>				
12	23	66	2	..
13	27	25	7	1
14	51	25	39	2
15	35	64	31	11
16	41	50	41	29
17	17	17	17	11
18	16	9	16	9
19	5	6	5	6

TABLE XXXVI.

A comparative table of ossification of the carpal phalanges.

Phalanges:—		1		2		3		4		5	
		M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
Cunningham	.. {	App.	3	3	3	3	3	3	3	3	3
		Fus.	18 to 20	18 to 20	18 to 20	18 to 20	18 to 20	18 to 20	18 to 20	18 to 20	18 to 20
Paterson	.. {	App.	3	2	3	2	3	2	3	2	2
		Fus.	19	17	19	17	19	17	19	17	17
Flecker {	App.	3	2	2	2	2	2	2	2	2
		Fus.	16/17	15/16	17	16	17	16	18	17	16
Sidhom <i>et al.</i>	..	Fus.	17/19	..	17/19	..	17/19	..	17/19	..	17/19
Galstaun, proximal row.	{	App.	2/4	1 ^a / ₁₇	2/4	1 ^a / ₁₇	2/4	1 ^a / ₁₇	2/4	1 ^a / ₁₇	2/4
		Fus.	17/18	14/15	17/18	14/15	17/18	14/15	17/18	14/15	17/18
Middle row	.. {	App.	3	3	3	3	3	3	3	3	3
		Fus.	16/18	14/16	16/18	14/16	16/18	14/16	16/18	14/16	16/18
Terminal row	.. {	App.	3	3/5	3	3/5	3	3/5	3
		Fus.	15	17/18	15	17/18	15	17/18	15

THE SESAMOID BONES.

The sesamoid of the thumb was seen in one girl aged 9, an average age of appearance is 12. It was not seen in boys till the age of 12 and was not consistently present till the age of 16.

The other carpal sesamoids were found to be so irregular in their appearance that they were not studied from a statistical standpoint.

The sesamoid of the great toe.—This was seen in five girls out of ten examined aged 9 and was present in all cases examined aged 12. In the case of boys the age of appearance varied from 9 to 16.

TABLE XXXVII.

The sesamoid (thumb).

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
8	10	32
9	10	15	1	..
10	17	41	1	..
11	4	44	1	..
12	21	63	17	11
13	20	25	17	6
14	27	25	27	14
15	19	64	19	35
16	28	50	28	50

TABLE XXXVIII.

The sesamoid (great toe).

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
8	10	32
9	10	15	5	1
10	17	41	8	3
11	4	44	2	10
12	21	63	21	32
13	20	25	19	18
14	27	25	27	21
15	19	64	19	60
16	28	50	28	50

OS INNOMINATUM.

Of the three components of the os innominatum, the earliest to commence ossification is the ilium in which a centre is seen at about the ninth week of intra-uterine life. The ischium ossifies next at about the fourth month. The

pubis is the last of the three bones to begin osseous development which starts from a centre anterior to the acetabulum between the fifth and sixth month.

At birth, and for the first few years of life, the three bony components are separated at the acetabulum by a triradiate piece of cartilage. The ischio-pubic junction is also separated by cartilage at the junction of the ascending portion of the ischial and the descending portion of the pubic ramus.

In addition to these main centres, accessory centres are found for the iliac crest, the tuber ischii, very occasionally for the anterior superior iliac spine and various portions of the triradiate cartilage mentioned above (os acetabuli). With regard to this latter it is extremely difficult in skiagrams to see the centrally-placed ossicles. One was not infrequently met with, however, at the upper edge of the acetabular lip (*see Appendix*).

THE CREST OF THE ILIUM.

The centre for the crest of the ilium was found to make its appearance at an average age of 14 in girls and 17 in boys. The earliest age at which it was seen in both girls and boys was 12. Fusion of this centre with the main mass of the ilium was found to take place at between 17 and 19 in girls and 19 and 20 in boys. It was found difficult to get a sufficient number of girls aged 17 and upwards for examination. This deficiency is in process of being remedied.

TABLE XXXIX.

The crest of the ilium.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
11	4	44
12	21	64	2	3
13	20	25	1	3
14	27	26	18	8	1	..
15	19	64	19	22	..	1
16	28	51	27	32	1	2
17	3	16	1	12	2	4
18	9	10	4	8	5	2
19	4	6	1	1	3	5
20	1	30	1	2	..	28
21	..	8	..	1	..	7
22	..	3	3

THE UNION OF THE ILIUM, ISCHIUM, AND PUBIS AT THE ACETABULUM.

The replacement of the triradiate cartilage of the acetabulum with bone was found to take place at between the ages of 13 and 14 on an average in girls and at round about 15 to 16 in boys. These figures are rather later than those given by Flecker who gives between 13 and 14 as the average age for girls and 15 for boys.

TABLE XL.

The union of the ilium, ischium, and pubis at the acetabulum.

Age.	Number of girls examined.	Number of boys examined.	Fusion of centres in girls.	Fusion of centres in boys.
10	17	43
11	4	44
12	21	64	7	4
13	20	27	8	4
14	27	25	25	9
15	19	64	19	27
16	23	50	28	40
17	1	16	1	13
18	7	10	7	10
19	2	6	2	6
20	..	29	..	29

THE UNION OF THE ASCENDING ISCHIAL WITH THE DESCENDING PUBIC RAMUS.

The union of these two bones was found to take place at an average age of $8\frac{1}{2}$ in both girls and boys. The earliest fusion was seen in two out of eleven boys aged 4. Three out of six girls aged 4 were found to have united.

TABLE XLI.

The union of the ascending ischial with the descending pubic ramus.

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
3	4	3
4	6	11	3	2
5	8	8	4	4
6	5	8	2	4
7	6	20	2	13
8	9	33	6	32
9	10	16	10	15
10	17	43	17	40
11	4	44	3	44
12	21	64	16	45
13	20	27	20	27
14	27	25	27	23
15	19	64	19	64
16	28	50	28	49
17	1	16	1	16
18	7	10	7	10

THE SYMPHYSIS PUBIS.

The epiphyseal centre for the symphysis pubis, such as is usually described in textbooks of anatomy, was not seen in any case in our series.

THE ISCHIAL TUBEROSITY.

This centre is seen as a thin plate of bone lying along the ascending ramus of the ischium and the descending ramus of the pubis. Stevenson described this as the ramal epiphysis as distinct from that of the tuber ischii. I have not been able to distinguish the two. The centre was found to appear at between 14 and 16 in girls and 16 and 18 in boys. It unites at round about 20 years. It is a difficult centre to be sure of as it is not always seen and it becomes a matter of conjecture to be sure as to whether it has not appeared, or appeared and united.

TABLE XLII.

The ischial tuberosity.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
13	20	25
14	27	25	14	2
15	19	64	15	..
16	28	51	22	18
17	3	16	2	6
18	8	10	7	9
19	4	6	3	4
20	..	30	..	8
21	..	8	..	7
22	..	3	..	1

THE FEMUR.

The ossification of the femur takes place from five centres usually, viz., shaft, head, greater and lesser trochanters, and lower end.

The shaft.—Ossification commences in the cartilaginous shaft early in the second month of foetal life. At birth a fairly well-ossified shaft is seen expanded at both ends.

The centre for the femoral head.—In three cases of each sex examined on the day of birth the centre for the head was not seen, nor was it seen in a girl of five weeks. In the case of a female child of four months the centre was seen to have just appeared, ossification being slightly more advanced on the left side than on the right. The centre on the right side was a mere speck just distinctly recognizable as an epiphysis. In the case of a male child of seven months no centre was seen on either side in one case, whereas it was present bilaterally in another. In a girl of the same age, the centre was seen on both sides, having just appeared. It was slightly larger on the left side.

Most authorities agree that the centre usually makes its appearance during the sixth to the twelfth month of life. Borovansky and Hnevkovsky report a case of a boy of three weeks in which the centre was seen.

All cases examined of both sexes of a year or more old showed appearance of the centre.

Union of the head of the femur with the shaft.—Two male cases were seen to unite at the age of 14, the majority uniting at the ages of 16 and 17.

In the case of females, two cases were seen to unite at the age of 13, and the majority at the ages of 14 and 15. At the latter age all cases examined were found to have united. These dates of union appear to be earlier than the figures given by other observers. Cunningham gives the age of fusion as between 18 and 20 years. Paterson gives 17 for females and 18 for males. Flecker gives 14 as the majority age for girls and 17 as the majority age for boys.

TABLE XLIII.

The epiphysis for the head of the femur.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3
Under 1 year.	7	6	3	3
1	5	7	5	7
2	7	11	7	11
3	4	3	4	3
4	6	12	6	12
5	8	9	8	9
6	5	8	5	8
7	6	20	6	20
8	9	33	9	33
9	10	16	10	16
10	17	43	17	43
11	5	44	5	44
12	21	64	21	64
13	20	27	18	27	2	..
14	27	25	11	23	16	2
15	19	64	..	54	19	10
16	28	50	..	28	28	22
17	3	16	..	5	3	11
18	8	10	8	10
19	4	6	4	6

THE EPIPHYSIS FOR THE GREATER TROCHANTER.

The centre for the greater trochanter was seen in a boy and a girl at the age of 3. It was absent in three out of six girls aged 4 and present in six out of twelve

boys at this age. It was present in all the cases examined, both boys and girls, at the age of 5.

Paterson gives the average ages of appearance as 5 in the case of males and 4 in the case of females. Flecker gives the age as 5 for both sexes and describes one case of a girl aged 2, in which the centre was present bilaterally.

The epiphysis for the greater trochanter has an irregular mottled appearance, not unlike that of a calcified lymph node which does not assume a regular outline until it is fairly well formed.

The majority ages for the fusion of this epiphysis were found to be 14 in the case of girls and 17 in the case of boys. All the girls examined had fused at the age of 15 and all the boys at the age of 18. Paterson gives the majority figure for boys and girls as 18 and 16 to 17 respectively. Flecker gives the average ages of union as 14 to 15 in girls and 17 to 18 in boys.

TABLE XLIV.

The epiphysis for the greater trochanter.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
At birth.	3	3
1	5	7
2	7	11
3	4	3	1	1
4	6	12	3	6
5	8	9	8	8
6	5	8	5	8
7	6	20	6	20
8	9	33	9	33
9	10	16	10	16
10	17	43	17	43
11	5	44	5	44
12	21	64	21	64
13	20	27	18	27	2	..
14	27	25	11	23	16	2
15	19	64	..	54	19	10
16	28	50	..	28	28	22
17	3	16	..	5	3	11
18	8	10	8	10
19	4	6	4	6
20	..	29	29

THE EPIPHYSIS FOR THE LESSER TROCHANTER.

The development of the lesser trochanter is not constant. In the great majority of cases during the first year a bud-like extension is seen from the shaft. In many cases this is the only source of development of the lesser trochanter. In other cases, at between 12 and 15, in both boys and girls, a separate centre of

ossification develops which fuses shortly after at an average figure of 15 to 17. These figures are more or less in agreement with those given by other authorities.

TABLE XLV.

The epiphysis for the lesser trochanter.

Age.	Number of girls examined.	Number of boys examined.	EXTENSION IN		APPEARANCE OF CENTRE IN		EXTENSION AND CENTRE.		FUSION IN	
			Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.
At birth.	3	3
Under 1 year.	7	6	1	2
1	5	7	1	7
2	7	11	4	9
3	4	3	4	0
4	6	12	2	10
5	8	9	7	6
6	5	8	4	4
7	6	20	2	9
8	9	33	5	19
9	10	16	4	9
10	17	43	8	24
11	5	44	3	26	..	1
12	21	64	9	26	3	4	1	6	5	5
13	20	27	1	14	9	5	4	..
14	27	25	7	9	..	4	4	4	15	3
15	19	64	..	24	..	17	..	5	19	10
16	28	30	..	9	..	7	..	7	28	20
17	3	16	1	..	2	3	11
18	8	10	8	10

THE DISTAL EPIPHYSIS OF THE FEMUR.

The centre of ossification for the lower epiphysis of the femur was seen in each of three boys and girls examined on the day of their birth. This is in agreement with the findings of other observers.

Union of the shaft was seen in two out of twenty-five cases in males aged 14. At the age of 16, sixteen out of fifty were united. All cases of 18 and more showed complete union.

In the case of females, sixteen out of twenty-seven had fused at the age of 14. At the age of 15, twenty-six out of twenty-eight had fused. At the age of 17 and more, all cases were found to have united.

Paterson gives the majority age of fusion in boys as 18 years and in girls 16 years. Flecker curiously finds the ages to be 19 and 17 respectively. His figures, however, show a relatively small proportion of cases examined.

TABLE XLVI.

The distal epiphysis of the femur.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3	3	3
Under 1 year.	6	3	6	3
1	5	4	5	4
2	6	12	6	12
13	20	25	20	25
14	27	25	11	23	16	2
15	19	64	1	59	18	5
16	28	50	2	34	26	16
17	1	16	..	6	1	10
18	7	10	7	10
19	2	6	2	6

THE TIBIA.

This bone is developed from three centres, one for the shaft and one for each end. Ossification commences in the shaft early in the second month of intra-uterine life. At birth the shaft is seen to be fairly well formed.

The proximal epiphysis of the tibia.—It is categorically stated in textbooks of anatomy and of medical jurisprudence that the centre of the proximal epiphysis of the tibia is present at birth. Pryor has described one case of a girl prematurely born at 8 months in whom he found the centre though it was absent in two others of the same age. This series includes three babies of each sex who were examined on the day of their birth. Each of the three girls was found to have the centre bilaterally. In one of the three boys the centre was definitely absent on both sides. In another it was doubtful on one side and absent on the other. The third had the centre present on both sides. It is certain that this epiphysis must be present very shortly after birth in every case.

Cunningham describes an accessory centre appearing just below the main one. We have not seen this centre in any case in our series. Very often a triangular-shaped projection towards the shaft is seen.

Fusion of the proximal tibial epiphysis with the shaft was found to take place in girls at the age of 14, the majority age being between 14 and 15. In boys only two cases were found to have united out of twenty-five at the age of 14, the majority age being 16, at which twenty-six out of fifty had united. In every case aged 18, this centre was found to have united with the shaft. In girls at the age of 16 only one out of twenty-eight remained ununited and all cases aged 17 or more showed complete union.

The following is a comparison of figures for the majority ages of union of this epiphysis :—

				Girls.	Boys.
Paterson	16 to 17	18 to 19
Flecker	15	18
Galstaun	14 to 15	16 to 17

TABLE XLVII.

The proximal epiphysis of the tibia.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3	3	2
Under 1 year.	6	3	6	3
1	5	4	5	4
2	6	12	6	12
13	20	25	20	25
14	27	25	15	23	12	2
15	19	64	4	59	15	5
16	23	50	1	24	27	26
17	1	16	..	6	1	10
18	7	10	7	10
19	2	6	2	6

THE CENTRE FOR THE TIBIAL TUBEROSITY.

The ossification of the tibial tuberosity is not always very regular. Any one, or a combination, of three things may take place. The centre of ossification may appear in the cartilage covering the upper anterior surface of the tibia which may unite in the ordinary manner. In the vast majority of cases a tongue of bone develops as a bud-like projection downwards from the proximal epiphysis of the tibia. This tongue which lies in cartilage may fuse with the adjacent shaft. In the majority of cases a combination of these two things appears to take place. Two cases examined in our series showed the presence of the osteochondritis described by Osgood and Schlatter. The discovery was accidental. The earliest ages at which the tongue was seen were 8 in the case of females and 9 in the case of males, the majority ages being 12 and 14 in both sexes.

The separate epiphysis described made its earliest appearance at the age of 9 in girls and 10 in boys. It is often a matter of difficulty in these cases to ascertain clearly whether the centre has or has not been incorporated with the tongue and formed one homogeneous mass.

TABLE XLVIII.
The tibial tuberosity.

Age.	Number of girls examined.	Number of boys examined.	TONGUE IN		CENTRE IN		TONGUE AND CENTRE IN		FUSION IN	
			Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.
8	9	32	1
9	10	16	1	1	1
10	17	42	1	..	3	2	1
11	4	44	1	..	1	..	1
12	21	63	13	11	2	8	3	4	..	1
13	20	25	11	6	2	6	4
14	27	25	9	12	..	5	2	..	9	2
15	19	64	1	28	..	9	1	5	17	9
16	28	50	..	20	..	2	..	1	28	25
17	1	16	..	2	1	13
18	7	9	7	9
19	2	6	2	6

THE DISTAL EPIPHYSIS OF THE TIBIA.

Ossification of the lower tibial epiphysis commences early, usually late during the first year of life. It was seen in all cases examined of both boys and girls at the age of 1, and the earliest appearance was in a girl aged 7 months. Borovansky and Hnevkovsky found it in one case of a boy aged 3 months and 3 weeks. Flecker found it in a girl of 7 and a boy of 9. As is usual with an epiphysis that appears early, fusion is relatively late. Union was found in two out of twenty-five boys at the age of 14 and one out of twenty girls at the age of 13. The majority ages were found to be 16 in boys and 14 to 15 in girls. In all girls aged 17 or more fusion was observed, whereas in boys the same was not seen until the age of 18.

A comparison of the average ages of fusion given by Paterson, Flecker, and myself is interesting:—

				Girls.	Boys.
Paterson	16	18
Flecker	14.1-3	17
Galstaun	14.1-4	16

TABLE XLIX.

The distal epiphysis of the tibia.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres ¹ in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3
Under 1 year.	5	3	3
1	3	4	3	1
2	7	8	7	8
13	20	26	19	26	1	..
14	27	25	14	23	13	2
15	19	64	1	56	18	8
16	28	50	1	27	27	23
17	1	16	..	6	1	10
18	7	9	7	9
19	2	6	2	6

THE FIBULA.

This bone ossifies from three centres, one for the shaft and one for each end.

The shaft.—The centre for the shaft begins to appear at about the sixth week of foetal life. At birth the bone is distinctly seen.

Proximal epiphysis of the fibula.—In our series of cases this epiphysis was seen at the age of 2 years in the case of girls and at the age of 4 years in the case of boys.

Union was seen in both boys and girls at the age of 14, the majority ages being 16 in both sexes.

Paterson found the majority ages for fusion in girls and boys respectively as 16 and 18 years. Flecker curiously found them both a year later. His total number examined in this series is not, however, very large.

TABLE L.

The proximal epiphysis of the fibula.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3
Under 1 year.	6	3
1	5	4
2	6	12	1
3	4	3	1
4	4	8	4	4

TABLE L—concl'd.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
5	7	5	6	4
6	5	7	4	3
7	7	20	7	18
8	9	33	9	32
14	27	25	23	24	4	1
15	19	64	10	62	9	2
16	28	50	9	38	19	12
17	1	16	..	9	1	7
18	7	10	..	3	7	7
19	2	6	2	6

THE DISTAL EPIPHYSIS OF THE FIBULA.

This centre was found to appear late during the first year of life in both sexes. It was present in a female child of 7 months. Union of the centre with the shaft was found at the age of 13 in females and 14 in males, the majority ages being 15 in females and 16 in males.

Paterson and Flecker both agree on 17 being the majority age of union in boys. In the case of girls, Paterson gives the figure of 16, while Flecker gives 14. He shows, however, a very small number of cases to support this figure, twenty-six in all including the ages of 14 to 17.

TABLE LI.

The distal epiphysis of the fibula.

Age.	Number of girls examined.	Number of boys examined.	Appearance of centres in girls.	Appearance of centres in boys.	Fusion of centres in girls.	Fusion of centres in boys.
At birth.	3	3
Under 1 year.	5	3	2
1	3	4	3	2
2	7	8	7	6
13	20	26	19	26	1	..
14	27	25	18	23	9	2
15	19	64	2	58	17	6
16	28	50	2	29	26	21
17	1	16	..	10	1	6
18	7	9	7	9
19	2	6	2	6

THE PATELLA.

The centre for the patella was seen in one out of three boys aged 3, and fifteen out of nineteen aged 7. It was constantly present from the age of 8 onwards. In the case of girls the patella was constantly present from the age of 4 onwards.

A double centre of ossification was sometimes seen in this bone.

Two cases were seen in this series of bipartite patella. The smaller component as usual was present at the supero-lateral aspect.

THE FABELLA.

No true fabella was seen in this series—that is to say a sesamoid bone in the gastrocnemius tendon presenting an articular surface with the posterior surface of the lower end of the femur. Two such cases have been seen by the author in the course of routine work.

A sesamoid bone in the gastrocnemius tendon was frequently met with and is a commonplace in skiagrams of adult knee joints.

TABLE LII.

The patella.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
Under 1 year.	6	3
1	5	4
2	6	12
3	4	3	..	1
4	4	9	4	3
5	7	5	7	5
6	5	7	5	3
7	7	19	7	15
8	9	33	9	33
9	10	16	10	16

THE TARSAL BONES.

All of the three cases of boys and girls examined at birth showed the presence of the calcaneum and talus.

In one out of three girls examined the cuboid was not seen ; it was present in all the other cases in both boys and girls.

The internal cuneiform was seen at birth in one out of three girls and two out of three boys.

The external cuneiform was seen in one out of five girls under 1 year. It was present in all the girls examined at the age of 3, and six out of seven boys aged 4.

The middle cuneiform bone was seen in one girl out of five under the age of 1 year and was constantly present in girls aged 3. In boys it was seen in six out of eight aged 2, and all the cases examined aged 4.

The navicular bone is usually the last of the tarsal bones to appear. Though it was seen in one out of five girls under 1 year, it was not constantly seen till the age of 3. In boys, four out of eight aged 2 showed the bone.

TABLE LIII.

The navicular.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
Under 1 year.	5	3	1	..
1	4	3	1	0
2	7	8	3	4
3	4	5	4	4
4	4	7	4	6
5	6	5	6	5

TABLE LIV.

The cuboid.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3	2	3
Under 1 year.	5	3	4	3
1	4	3	3	4
2	7	8	7	8
3	4	5	4	5
4	4	7	4	7

TABLE LV.
The internal cuneiform.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3	1	2
Under 1 year.	5	3	3	0
1	4	3	3	2
2	7	8	7	4
3	4	5	4	4
4	4	7	4	7

TABLE LVI.
The middle cuneiform.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
Under 1 year.	5	3	1	..
1	4	3	0	..
2	7	8	2	6
3	4	5	4	3
4	4	7	4	7
5	6	5	6	5

TABLE LVII.
The external cuneiform.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
At birth.	3	3
Under 1 year.	5	3	1	..
1	4	4	2	2
2	7	8	1	2
3	4	5	4	2
4	4	7	4	6
5	6	5	6	5

THE APOPHYSIS OF THE CALCANEUM.

The apophysis of the calcaneum was seen to have appeared in two girls aged 6 and two boys aged 7, the majority age for girls and boys respectively being 7 and 9 years.

This centre is often fragmented and irregular in appearance.

TABLE LVIII.

The apophysis of the calcaneum.

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.	Fusion in girls.	Fusion in boys.
6	5	6	2
7	7	19	4	2
8	9	32	8	8
9	10	15	10	7
10	17	41	17	26
11	4	44	4	36
12	21	65	11	64	10	1
13	20	26	14	24	6	2
14	27	25	5	20	22	5
15	19	64	..	40	19	24
16	28	50	..	13	28	37
17	1	16	1	16
18	7	9	7	9
19	2	6	2	6

THE METATARSALS.

The mode of ossification of the metatarsals is very similar to that of the metacarpals. The first metatarsal behaves in exactly the same way as the corresponding metacarpal, and centres for the second, third, fourth, and fifth metatarsals appear in exactly the same way. The primary centres in each case, as in the metacarpals, appear round about the ninth week of foetal life.

The centres for the epiphyses of the metatarsals appeared at an average age of 3 in girls and 4 to 5 in boys. The first metatarsal seemed to appear rather earlier.

Union of the epiphysis with the diaphysis in the metatarsals was seen to take place at an average age of 14 to 15 in girls and 16 to 18 in boys.

A separate distal epiphysis for the first metatarsal was seen in only one out of six hundred cases in a boy aged 10.

TABLE LIX.

The metatarsals.

Age.	Number of girls examined.	Number of boys examined.	APPEARANCE IN GIRLS.					APPEARANCE IN BOYS.					REMARKS.
			I.	II.	III.	IV.	V.	I.	II.	III.	IV.	V.	
2	7	8	1	1	1	1	1	1	1	1	No centre had appeared under 2 years.
3	3	5	3	3	3	2	..	4	1	1	1	1	
4	4	7	4	4	4	4	3	7	5	3	3	1	
5	6	4	6	6	6	6	5	4	4	4	3	2	
6	5	6	5	5	5	5	5	6	5	4	4	4	
7	17	17	17	17	17	

TABLE LX.

The metatarsals—union.

Age.	Number of girls examined.	Number of boys examined.	FUSION IN GIRLS.					FUSION IN BOYS.				
			I.	II.	III.	IV.	V.	I.	II.	III.	IV.	V.
12	21	64	3	3	3	3	3	2	3	3	3	..
13	20	25	6	5	5	6	5	1	3	3	3	..
14	27	25	18	18	18	18	16	2	4	4	4	2
15	19	64	17	17	17	17	17	12	16	14	14	9
16	28	50	27	27	27	27	27	29	33	33	33	30
17	1	16	1	1	1	1	1	11	13	14	14	11
18	7	9	7	7	7	7	7	9	9	9	9	9
19	2	6	2	2	2	2	2	6	6	6	6	6

THE TARSAL PHALANGES.

These again ossify in exactly the same manner as the phalanges of the hand. The primary centres for the ungual phalanges appear first at the eleventh to the twelfth week of foetal life. Those for the first row appear from the fourteenth to the sixteenth week. In the case of the middle phalanges of the second and third toe, ossification commences about the sixth month and those for the fourth and fifth toes, a short time later. The ossification for the fourth and fifth toes in the middle and terminal phalanges is frequently delayed till after birth. The epiphyseal centres in the case of the proximal phalanges appear between the first and third year in girls and between the second and fourth in boys. They fuse with the shafts at between 14 and 15 in the case of girls and 16 and 18 in the case of boys.

The epiphyses for the middle phalanges appear at between the ages of 3 and 4 in girls and boys. They unite at between the ages of 14 and 15 in girls and 16 and 17 in boys.

The epiphyses for the terminal phalanges appear at between 4 and 6 in both girls and boys. They fuse at between the ages of 13 and 14 in girls and 15 and 17 in boys.

TABLE LXI.

The phalanges (feet).

Age.	Number of girls examined.	Number of boys examined.	Appearance in girls.	Appearance in boys.
<i>Proximal.</i>				
1	3	4	2*	0
2	7	8	2*	2*
3	4	4	4†	3† *
4	4	7	4	7
5	6	4	6	4
6	5	6	5	5
<i>Middle.</i>				
2	7	8
3	4	4	2† 1 All	2§
4	4	7	3† 1 All	7
5	6	4	6	4
6	5	6	5	5
<i>Terminal.</i>				
2	7	8
3	4	4
4	4	4	4	2
5	6	4	5	3
6	5	6	5	3

* Except great and little toes.

† Except 4th and 5th digits.

‡ Except little toe in one case.

§ Except 1st, 4th, and 5th digits

TABLE LXII.

The phalanges (feet).

Age.	Number of girls examined.	Number of boys examined.	Fusion in girls.	Fusion in boys.
<i>Proximal.</i>				
12	21	64	1	..
13	20	25	5	2
14	27	25	18	2
15	19	64	17	10
16	28	50	28	30
17	1	16	1	10
18	7	9	7	9
19	2	6	2	6
<i>Middle.</i>				
12	21	64	5	..
13	20	25	7	1
14	27	25	21	3
15	19	64	17	16
16	28	50	28	36
17	1	16	1	13
18	7	9	7	9
19	2	6	2	6
<i>Terminal.</i>				
12	21	64	6	..
13	20	25	11	2
14	27	25	25	6
15	19	64	17	23
16	28	50	28	35
17	1	16	1	13
18	7	9	7	9
19	2	6	2	6

Flecker has described the os vesalii as the non-united epiphysis for the tuberosity of the fifth metatarsal bone. We cannot agree with this view. The os vesalii seen in our series was always proximal to this tuberosity. This is in agreement with Köehler, and also with Baastrup (1922).

The epiphysis at the base of the fifth metatarsal was found in two positions, in some it lay laterally and in others almost at the tip. Fractures are often found here, but as all our cases examined were done especially with a view to investigating ossification and not as a result of an injury, this has been ruled out. With regard to the more proximally placed os vesalii, the description which I take the liberty of quoting from Köehler (*loc. cit.*) is illuminating.

'At the proximal end of the fifth metatarsal bone there occur two epiphysial-like forms. (1) The "apophysis", a frequent, perhaps constant, scale-like epiphysis

at the lateral plantar part of the tuberosity of the fifth. Its ossification begins in the tendon of the peroneus brevis muscle. (2) The proximal part of the tuberosity of the fifth has occasionally a certain independence with a special ossification centre. Occasionally there occurs an epiphysal-like terminal form, which in three cases proved at the autopsy to be not epiphyses at all, but independent bones, the os vesalianum tarsi. The tuberosity of the fifth metatarsal bone corresponds morphologically, phylogenetically, and ontologically with the fifth os tarsali that has disappeared in the distal row, and the os vesalianum is to be regarded as an atavistic form of the fifth os tarsali. The fact that the os vesalianum is found so seldom (although it occurs more frequently than is generally believed) leads one to think that the fifth os tarsali disappears in an earlier stage of development. With such a theory agrees also the fact, that a fifth os tarsali is found only in anura, reptiles, and in embryonic life of the lowest classes of the mammalia. The theory is supported by the varying position of the bones in the early stages of the human embryo. Occasionally the os vesalianum and the above-mentioned apophysis is taken for a fracture of the tuberosity of the fifth metatarsal. The history and the Röntgen pictures must decide the differential diagnosis. The usual criterion advanced for determining real bones, namely, their bilaterality, does not assist us to a correct diagnosis.'

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APPENDIX.

Age.	Anomaly, accessory ossicle, or unusual epiphysis met with.	Females (number).	Males (number).
8	Bipartite patella	1
	Accessory centre for the tip of the lateral malleolus	..	1
	Accessory centre for the tip of the medial malleolus	2	..
	Os vesalii	2	..
10	Accessory centre for the tip of the lateral malleolus	..	1
	Accessory centre for the tip of the medial malleolus	3	5
	Accessory centre for the tip of the base of the Vth metatarsal.	3	..
	Os naviculare accessorius	1	..
11	Accessory centre for the tip of the medial malleolus	1	5
	Accessory centre for the ulnar styloid process ..	1	2

APPENDIX—concl'd.

Age.	Anomaly, accessory ossicle, or unusual epiphysis met with.	Females (number).	Males (number).
12	Accessory centre for the tip of the lateral malleolus	..	1
	Accessory centre for the tip of the medial malleolus	..	7
	Accessory centre for the tip of the base of the Vth metatarsal.	..	4
	Accessory centre for the upper lateral edge of the acetabular lip.	..	2
	Os naviculare accessorius	1
13	Accessory centre for the tip of the medial malleolus	..	3
	Accessory centre for the base of the Vth metatarsal	2	5
14	Accessory centre for the tip of the base of the Vth metatarsal.	1	3
	Accessory centre for the upper lateral edge of the acetabular lip.	1	1
	Os naviculare accessorius	2	..
15	Accessory centre for the tip of the base of the Vth metatarsal.	..	10
	Bipartite patella	1
	Os trigonum	1
	Accessory centre for the upper lateral edge of the acetabular lip.	..	2
	Os naviculare accessorius	1	..
16	Accessory centre for the tip of the base of the Vth metatarsal.	..	4
	Os naviculare accessorius	3	1
	Os trigonum	1
	Accessory centre for the upper lateral edge of the acetabular lip.	..	4
17	Accessory centre for the upper lateral edge of the acetabular lip.	..	1
20	Os naviculare accessorius	1
22	Os vesalii	1

NOTES ON A NEW STRAIN OF ACTINOMYCES OBTAINED BY BLOOD CULTURE.

BY

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[Received for publication, April 29, 1937.]

Source.—The strain was obtained by blood culture from a patient, with high fever and patches of bronchopneumonia in both the lungs. The case was reported by Ghosh (1936). The blood culture was done on the ninth day of disease.

Cultural reactions.—The organism grows easily in all artificial media, under aerobic conditions, at ordinary room temperature and in the incubator.

Nutrient agar.—Dull white colonies of varying size, strongly adherent to the surface of agar, due to the mycelia growing into the substratum.

Glucose agar.—Similar to growth on nutrient agar, but shows some wrinkling on the surface of the colonies in six days.

Blood agar.—Similar to growth on nutrient agar but the colonies are smaller. No hæmolysis.

Synthetic agar.—Greyish-green aerial mycelia very loosely attached to the surface, becoming brown in three days.

Egg medium.—Thick wrinkled skin, becoming greenish in six days. Aerial mycelia not formed in moist media but starts forming when the medium becomes dry. Four per cent glycerine added to the medium inhibits the growth of the organism.

Potato.—Heaped up wrinkled greenish mycelia covered with white powdery growth, becoming brown in 15 days.

Starch agar.—Copious aerial mycelia formed. Shows white powdery growth in 48 hours, slightly adherent to the surface, later on becoming brown (Plate XII, fig. 1).

Gelatine stab.—No liquefaction. Greenish wrinkled growth on the surface, none in the depth.

Tellurite medium.—Growth scanty, colonies slightly dark.

MacConkey agar.—No growth.

326 *A New Strain of Actinomyces obtained by Blood Culture.*

Glucose broth.—Small puff-ball-like growth at the bottom of the tube. When planted on floating pieces of cork in the broth flask, a greenish felted mass covered with a whitish powdery growth is seen in a week to ten days.

Litmus milk.—Slightly acid in five days, strongly acid in eight days. Milk is digested and the medium looks a clear red fluid with a slight deposit at the bottom. At the same time a thick white ring, slightly green at the edges, forms on the surface of the medium.

Sugar reactions.—Lactose, saccharose, mannite, and maltose are not fermented. A very slight acidity occurs in glucose in seven days.

Other biochemical reactions.—Methylene blue, slightly reduced, becomes greenish in three days. Methyl red slightly positive. Nitrites formed from nitrates. Ammonia formation slight. H_2S not formed. Amylase and catalase formed. Indol a bare trace in seven days.

Morphology.—Smears from young agar cultures show Gram-positive branching filaments, measuring 0.2μ to 0.5μ in thickness and are not acid-fast. Smears from old agar and broth cultures show Gram-negative or partly Gram-positive and partly Gram-negative filaments. Beaded filaments are also seen, the beads are Gram-positive and the intervening filaments Gram-negative. No acid-fast 'spores' are seen in smears made from agar or blood-agar cultures. Smears from starch and synthetic agar cultures, however, show acid-fast 'spores'. Impression preparations (Plate XII, fig. 2), made from a four-day-old culture on rice agar, show the mode of formation of these 'spores'. Such preparations show a large number of beaded filaments, a good number of which are acid-fast. As the cultures become old and the growth becomes brown, smears show acid-fast coccoid 'spores' in a free state with a few stray filaments, which are not acid-fast. The 'spores' are slightly oval measuring about $1.2\mu \times 0.6\mu$. They are acid- and alcohol-fast. They are not decolorized by 25 per cent sulphuric acid in one minute, and a good proportion is not decolorized in five minutes. They have a tough coat, are not wetted by water, and are stained very faintly by ordinary stains. They do not take Gram's stain as gentian violet cannot penetrate through the outer coat. They are more heat-resistant than the filaments. The filaments are killed at $56^\circ C$. in half an hour, but some of the granules can stand $80^\circ C$. for five minutes. Boiling for five minutes kills them all. When planted on agar, they germinate, and branching filaments can be seen growing out of these granules. On germination the 'spores' cease to be acid-fast and become pervious to all dyes.

ANIMAL INOCULATION.

Intravenous, subcutaneous, and intraperitoneal inoculations into rabbits produced no lesions. Intratesticular injections of broth culture did not give any result.

NOMENCLATURE.

This organism appears to belong to a new species as it differs from all the species described by Bergey (1930), under the head of species pathogenic to animals. The noteworthy point about the organism is the formation of aerial mycelia on synthetic and starch agar, egg and potato and subsequent formation

PLATE XII.

Actinomyces sanguinis n. sp. Basu.

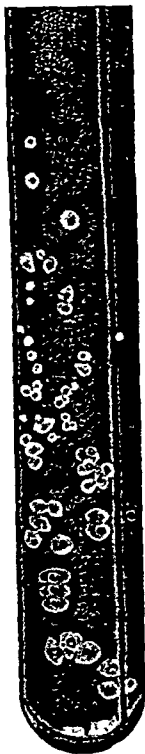


FIG. 1

Starch agar 7 days.

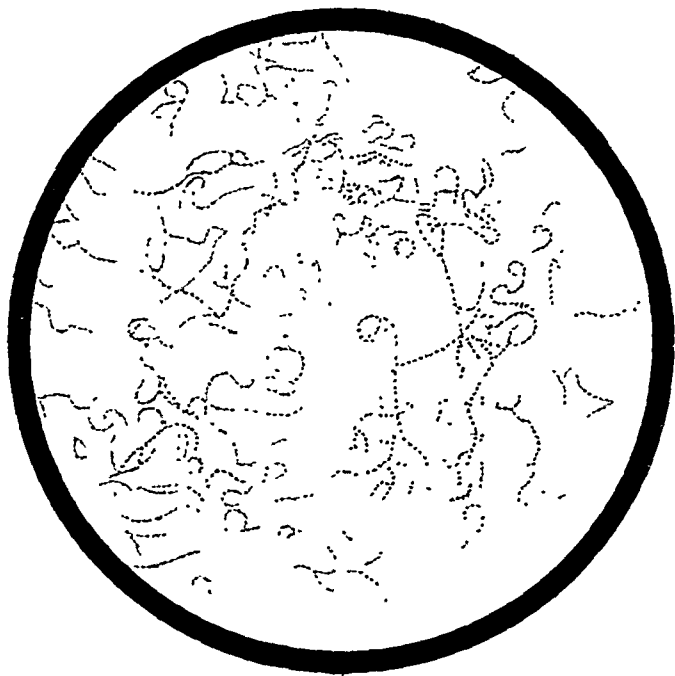


FIG. 2.

Impression preparation of 4 days old culture on rice agar stained by Ziehl and Neelsen method.

of acid-fast spores, while cultures on nutrient agar and blood agar give rise to vegetative hyphæ only. Actinomyces have been known to metastase in distant organs but so far I have not come across the record of any case of septicæmia produced by actinomyces with the probable exception of one reported by Scudder (1932). Unfortunately no detailed study of the organism has been published by the author. From the fact that the present organism was isolated from blood I propose to place it under a new species to be called *Actinomyces sanguinis*.

Cultures of the organism were sent to Dr. R. St. John Brooks, Curator of the National Collection of Type Cultures, Lister Institute, London, who very kindly sent it to Miss D. Erikson of the London School of Tropical Medicine and Hygiene for opinion. She is also of the opinion that this organism does not agree exactly with any pathogen described in literature. Her note is appended to the paper.

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APPENDIX.

NOTE ON ACTINOMYCES SANGUINIS N. SP. BASU.

BY

D. ERIKSON.

FROM the description of the author and confirmatory evidence obtained by study of the specimen sent, this organism belongs to Group I ørskov: 'Sporogenous fungi, the spores growing into a unicellular mycelium which shows no spontaneous division'.

Within this group the organism appears to come under Section A: 'No soluble pigment on protein media, proteolytic action weak or absent' (Erikson). It is a remarkable point that gelatine is not liquefied and in this respect it resembles *A. horton* (N. C. T. C. No. 600), but differs from this strain by its much more abundant growth on synthetic media.

The acid-fastness of the spores, in contrast to the non-acid-fastness of the vegetative hyphæ, is more marked on synthetic media. This phenomenon, however, does not seem to me to merit specific rank, since I have found the same thing to obtain with several of my presumed acid-fast strains, and it has been pointed out by Lieske that there is always a general tendency among the actinomyces for the quality of acid-fastness to be retained more specifically by the spores.

The abundant greyish-green aerial mycelium produced on synthetic media brings this form nearer some of the soil strains, cf. *A. lipmannii* Waksman, from which, nevertheless, it differs by its poorer proteolytic properties.

As far as I know, this strain does not agree exactly with any pathogen described in the literature and consequently merits careful description.

A STUDY OF *M. LEPRÆ MURIS* IN TISSUE CULTURES AND IN CHICK-EMBRYO MEDIUM.

BY

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INTRODUCTION.

WORKERS on the bacteriology of leprosy may be divided into three main groups made up as follows :—

1. Those who have not been able to isolate any organism from leprous lesions.
2. Those who have isolated from leprous lesions an organism which is under certain conditions acid-fast and under other conditions not acid-fast.
3. Those who have isolated from leprous lesions organisms which are persistently acid-fast.

Regarding the first group, which includes the present writers, we need say nothing except that it is by far the largest of the three groups.

The theories of the second group were apparently originated by Barrinikow in 1899 but the chief exponents of this theory have been Kedrowsky and Bayon.

Kedrowsky has advocated these views from 1900 till the present time and has produced experimental evidence in support of them. As early as 1900 he isolated

an acid-fast diphtheroid and observed the loss of acid-fast properties of the organism in old cultures and the change of these non-acid-fast organisms into acid-fast organisms when inoculated into rabbits. He was of the opinion that the acid-fast property was acquired only in the body. In 1910 he isolated non-acid-fast diphtheroids from a skin nodule and was able to change them into acid-fast types by inoculation into guinea-pigs and rabbits. Kedrowsky (1928) cites experiments to show that with the changes in the media and laboratory environment diphtheria and diphtheroid bacilli could be changed into thread fungi, and that thread fungi could be changed into organisms resembling diphtheroids. From these experiments and from his previous work of converting non-acid-fast organisms isolated from leprosy lesions into acid-fast ones and vice versa, he concludes that the leprosy bacillus when placed in unfavourable surroundings can undergo considerable changes, that these changes may cause the bacillus to lose its acid-fast property and to be changed into a thread fungus closely resembling the ordinary actinomyces. According to Kedrowsky, Hansen's bacillus as we know it represents only one of its 'states of being'.

Kedrowsky's chief supporter has been Bayon (1911) who reported similar findings. Other workers, who have isolated facultative acid-fast bacilli from leprosy lesions, include Williams (1911), Liston and Williams (1912), Johnston (1917), Walker (1929), Walker and Sweeney (1935*a* and *b*), Vaudremer, Sezary and Brun (1932). Vaudremer and Brun (1935), Löwenstein (1935), Salle (1934), and Kriz (1936). The organisms isolated by these workers by various methods and on various media have been chiefly diphtheroids, streptothrices, and coccoid organisms.

The third group of workers has isolated from leprosy lesions organisms which are persistently acid-fast. This group includes such workers as Clegg (1909), Duval (1910), Twort (1910), Wherry (1930), Timofejewsky (1929), McKinley and Soule (1932), Soule and McKinley (1932), McKinley and Verder (1933*a*), and Soule (1934). Most of these workers do not consider that the facultative acid-fast bacilli isolated by the second group are really the bacillus of leprosy. Special mention may be made of the work of Duval, Timofejewsky, McKinley and Soule, Soule and McKinley, and McKinley and Verder. These workers have during the last few years by modern bacteriological methods of various kinds isolated from leprosy lesions bacilli which are persistently acid-fast. Soule and McKinley claim to have maintained their acid-fast organism through 26 generations over a period of two and a half years without ever observing any change in morphology or staining reactions of the organisms. Duval (1934) also has studied the morphology and staining reactions of bacilli in pieces of leproma seeded on suitable media over a period of six months, and found only minor changes in morphology and no changes in staining reaction.

MEDIA CONTAINING LIVING TISSUE.

The findings of Salle (*loc. cit.*) and Walker and Sweeney (1935*a*) have once more raised the question as to whether the organism of leprosy is persistently acid-fast under all circumstances or is only a facultative acid-fast bacillus. These workers have in tissue cultures and in minced chick-tissue medium grown facultative acid-fast bacilli from lesions of human and rat leprosy. We therefore propose

in this paper to quote the publications of other workers, and to give details of our own work on the use of these media for the cultivation of the leprosy bacillus.

The possibility of the application of tissue-culture methods to the problem of the culture of the bacillus of leprosy attracted some attention during the last twenty-five years. The first attempt to follow this line of work was made by Zinsser and Carey (1912). Similar work was done by Fraser and Fletcher (Fraser, 1914). The first two workers reported multiplication of bacilli, but the second two failed to verify this. With recent improvements in tissue-culture technique, the matter has been studied again by Friedheim, Timofejewsky, Soule, and Salle. The findings of these workers are summarized later.

Media prepared with minced chick-embryo have also been used for the cultivation of the bacillus of leprosy. The first reference on the subject is contained in the annual report for the year 1932 of the Surgeon-General of the Public Health Services of the United States. Various workers including McKinley and Verder, Soule, Walker and Sweeney, and Holt (1934) have tried to grow the leprosy bacillus in this medium with varying results which are quoted later.

During the last three years we have in this department been experimenting with similar methods in the isolation of the bacillus of leprosy. The work described in the present paper has been done chiefly by one of us (D.) during the last year but the other worker has kept in close touch with the work and has verified the findings which are in complete agreement with the unpublished findings of previous work done along these lines by himself personally. We have preferred to use the organisms of rat leprosy for this work because rat tissues and embryos are easily obtainable and also because the genuineness of any organisms isolated can be proved by animal inoculation. In this paper we propose to describe our attempts to study the rat-leprosy bacillus in tissue culture and minced tissue medium using emulsions from spleens of rats suffering from rat leprosy experimentally produced. The emulsions used were always rich in acid-fast bacilli and in some of the experiments the emulsion was treated with 5 per cent sulphuric acid for five minutes to kill any contaminating organisms, and then neutralized.

TISSUE CULTURE.

The following results have been reported :—

1. No growth of bacilli obtained. Fraser and Fletcher (Fraser, *loc. cit.*).
2. Growth of acid-fast organism. Timofejewsky (*loc. cit.*) claims to have obtained multiplication of the leprosy bacilli in cultures of fragments of leprosy nodules planted in human and rabbit plasma and extract of human embryo. The tissues died off after a few weeks but the bacilli increased for a time even after the tissue died. When the bacilli from such old tissue cultures were transferred to cultures of healthy human tissue and of leucocytes, multiplication of these bacilli was obtained. The only form of bacillus seen throughout the

experiments was the acid-fast rod often granular. Soule (*loc. cit.*) obtained slow but definite growth of acid-fast organisms.

3. Facultative acid-fast organisms grown. Salle (*loc. cit.*) isolated mixed cultures of acid-fast organisms and diphtheroids four times out of six trials with human-leprosy material and once out of two trials with rat-leprosy material.

His method was as follows: He took chick-embryo tissue, immersed it in an emulsion made from the leprosy lesions and then cultured the tissue in Carrel flasks containing heparinized guinea-pig plasma and chick-embryo extract. A growth of acid-fast and non-acid-fast organisms was obtained, and later this was transferred to chick-embryo medium, and to ordinary laboratory media. Salle summarizes his own findings as follows:—

‘Chick-tissue cultures, prepared according to the method of Carrel, were used as culture medium for the isolation of the organisms of human and rat leprosy.

‘An acid-fast organism and a diphtheroid were isolated from four human nodules and one rat granuloma.

‘When transfers were made to artificial culture mediums (egg, potato, agar, etc.) only the blue-staining diphtheroid multiplied.

‘After the primary isolations on chick-tissue cultures, minced chick-embryo medium furnished an excellent substrate for the cultivation of the organisms. As with chick-tissue cultures, the minced embryo medium gave rise to acid-fast and non-acid-fast organisms.

‘A pure culture of the diphtheroid when inoculated into chick-embryo medium gave acid-fast and non-acid-fast organisms.

‘The diphtheroid and the acid-fast rods are apparently different phases of growth of the same organism.

‘The organisms were acid-fast in tissues and non-acid-fast on laboratory mediums. The tinctorial characteristics varied depending on the living condition of the tissues. In vigorous, actively-growing tissue the organisms were strongly acid-fast. As the tissue became less vigorous, the acid-fast property was less pronounced and, finally, as the tissue died, only non-acid-fast diphtheroids were seen.

‘It is believed that human and rat leprosy are caused by one and the same organism.’

Present work.—In our experiments pieces of spleen and skin from rat embryos were cultured in chick-plasma and rat-embryo extract. It was found on several occasions that chick-plasma did not yield a good clot on the addition of rat-embryo extract and therefore a drop of chick-tissue extract was added to aid clotting. The pieces of tissues before being cultured were infected with the rat-leprosy bacillus by immersion for half an hour in the emulsion of bacilli. Some of the pieces were cultured without being infected. In the experiments here reported the cultures were kept in the incubator at 37°C. for seven days without being sub-cultured. (In other experiments we have sub-cultured the growing tissue infected with leprosy bacilli every three days for a total period of up to one month but we have obtained no proof of multiplication of organisms.) At daily intervals a number of these cultures were taken out and examined by making smears or sections. Two smears were made from each of the cultures examined by means of smears, one from the tissue itself and the other from the surrounding medium. In all, 47 cultures were thus studied, of these 15 pieces were uninfected and 32 were infected. Out of the 47 cultures, 30 were examined by making smears and 17 by making sections. The detailed results of the examinations are shown in Table I:—

TABLE I.

Results of bacteriological examination of tissue cultures.

Day of exami- nation.	15 UNINFECTED TISSUES.			32 TISSUES INFECTED WITH <i>M. lepræ muris</i> .			
	Number examined.	RESULTS.		Number examined.	RESULTS.		REMARKS.
		A. F.	N. A. F.		A. F.	N. A. F.	
1st ..	3	5	5
2nd ..	4	6	6
3rd ..	1	9	9	1	In 1 specimen both A. F. and N. A. F.
4th ..	3	5	4	..	In 1 specimen neither A. F. nor N. A. F.
6th ..	3	6	5	..	In 1 specimen neither A. F. nor N. A. F.
7th ..	1	1	1
TOTAL ..	15	32	30	1	In one specimen both A. F. and N. A. F. In two specimens neither.

A. F. = Acid-fast bacilli.

N. A. F. = Non-acid-fast bacilli.

No organisms of any kind were found in the cultures not seeded with rat-leprosy bacillus either by means of smears or sections.

SUMMARY OF FINDINGS IN TISSUES INFECTED WITH *M. lepræ muris*.

The following findings were made:—

1. Acid-fast bacilli were present throughout the seven days in all cultures except two which were negative on the fourth and sixth days.
2. There was no evidence of multiplication of acid-fast bacilli in the cultures.
3. The acid-fast bacilli were more numerous in the tissue than in the surrounding medium (which was an expected finding) and there was no difference in the staining properties of bacilli in the tissues and the surrounding medium.

4. Non-acid-fast bacilli were not found at all and in only one culture were non-acid-fast organisms seen; these were diplococci and were found equally in the tissue and in the surrounding medium. Acid-fast bacilli were also found in this culture.
5. The findings of Salle were thus not confirmed.

Further experiments on staining properties of bacilli.—To see further whether the acid-fast character of the bacilli is lost in dead tissue bacilli were seeded in dead tissue. The experiment took the following form: An emulsion rich in acid-fast bacilli was prepared from the spleen of a rat in the advanced stage of leprosy. This emulsion was treated with 5 per cent sulphuric acid for 15 minutes; it was then centrifugalized without being neutralized and the deposit was taken up in normal saline. Pieces of spleen and skin from rat embryos were immersed in this emulsion for half an hour and were then cultured in chick-plasma and chick-tissue extract. Owing to the acid character of the emulsion used the tissues did not grow. Twenty such cultures were examined at intervals of about a week for six weeks by means of smears and sections. In all the cultures examined acid-fast bacilli were seen and in not a single specimen were non-acid-fast organisms found. The results are shown in Table II:—

TABLE II.

Results of bacteriological examination of dead tissue seeded with M. lepræ muris.

Day of examination.	Number examined.	RESULTS.	
		A. F.	N. A. F.
8th ..	7	7	..
15th ..	4	4	..
22nd ..	3	3	..
29th ..	4	4	..
43rd ..	2	2	..
TOTAL ..	20	20	..

A. F. = Acid-fast bacilli.
N. A. F. = Non-acid-fast bacilli.

To study the variations in staining characters of the bacilli when kept without any tissues the following experiment was carried out: The drop of chick-plasma on a cover-slip was infected with an emulsion containing rat-leprosy bacilli, a drop of chick-tissue extract was next added, and mixed well with the infected plasma. When the plasma had clotted the cover-slip was inverted on a depression slide and sealed with paraffin as in the case of tissue cultures. Eight such specimens were

examined at intervals of about a week for four weeks by means of smears and sections. In all the specimens examined acid-fast bacilli were seen while no non-acid-fast organisms were observed (Table III).

TABLE III.

Results of bacteriological examination of the clot of chick-plasma and chick-tissue extract infected with M. lepræ muris.

Day of examination.	Number examined.	RESULTS.	
		A. F.	N. A. F.
4th ..	2	2	..
8th ..	3	3	..
15th ..	1	1	..
22nd ..	1	1	..
29th ..	1	1	..
TOTAL ..	8	8	..

A. F. = Acid-fast bacilli.

N. A. F. = Non-acid-fast bacilli.

Conclusions.—Thus, working with tissue cultures, we were not able to confirm the findings of Salle and Soule regarding the multiplication of the leprosy bacillus in the tissue and in the medium or the findings of Salle regarding the difference in the staining characters of these bacilli in the living and in the dead tissues. (The tissue cultures in our experiments were kept without being sub-cultured for seven days, sub-culture after three or four days is essential to keep the tissue living. Thus if in dead tissue non-acid-fast forms developed, they should have been clearly seen by the seventh day.)

MINCED CHICK-EMBRYO MEDIUM.

With this medium also different workers have reported different results. The results of attempted cultivation of the leprosy bacillus can be grouped as under :—

1. No organism grown. Holt (*loc. cit.*) seeded *M. lepræ* on liquid and solid media containing chick-tissue but failed to get any growth.
2. Acid-fast organisms grown. McKinley and Verder (1933b) claim to have obtained a marked multiplication of bacilli in a few days in this medium. The cultures after being grown in this medium were transferred to laboratory media and in these media definite micro-colonies of acid-fast organisms were obtained. Soule (*loc. cit.*) confirmed the work of McKinley and Verder. Out of 26 attempts at cultivation of *M. lepræ* in the medium he obtained 'unquestioned evidence of proliferation in 22 instances'.

4. Non-acid-fast bacilli were not found at all and in only one culture were non-acid-fast organisms seen; these were diplococci and were found equally in the tissue and in the surrounding medium. Acid-fast bacilli were also found in this culture.
5. The findings of Salle were thus not confirmed.

Further experiments on staining properties of bacilli.—To see further whether the acid-fast character of the bacilli is lost in dead tissue bacilli were seeded in dead tissue. The experiment took the following form: An emulsion rich in acid-fast bacilli was prepared from the spleen of a rat in the advanced stage of leprosy. This emulsion was treated with 5 per cent sulphuric acid for 15 minutes; it was then centrifugalized without being neutralized and the deposit was taken up in normal saline. Pieces of spleen and skin from rat embryos were immersed in this emulsion for half an hour and were then cultured in chick-plasma and chick-tissue extract. Owing to the acid character of the emulsion used the tissues did not grow. Twenty such cultures were examined at intervals of about a week for six weeks by means of smears and sections. In all the cultures examined acid-fast bacilli were seen and in not a single specimen were non-acid-fast organisms found. The results are shown in Table II:—

TABLE II.

Results of bacteriological examination of dead tissue seeded with M. lepræ muris.

Day of examination.	Number examined.	RESULTS.	
		A. F.	N. A. F.
8th ..	7	7	..
15th ..	4	4	..
22nd ..	3	3	..
29th ..	4	4	..
43rd ..	2	2	..
TOTAL ..	20	20	..

A. F. = Acid-fast bacilli.

N. A. F. = Non-acid-fast bacilli.

To study the variations in staining characters of the bacilli when kept without any tissues the following experiment was carried out: The drop of chick-plasma on a cover-slip was infected with an emulsion containing rat-leprosy bacilli, a drop of chick-tissue extract was next added, and mixed well with the infected plasma. When the plasma had clotted the cover-slip was inverted on a depression slide and sealed with paraffin as in the case of tissue cultures. Eight such specimens were

The tubes showing no organisms were discarded; sub-cultures into fresh minced chick-tissue media tubes were made from some of the tubes in the other three groups. The results of these experiments are shown in Table IV:—

TABLE IV.

Bacteriological examination of chick-embryo medium seeded with M. lepræ muris.

Details of experiments.	Number of tubes inoculated.	RESULTS.			
		Organisms not found.	Acid-fast bacilli only found.	Acid-fast bacilli with non-acid-fast organisms.	Non-acid-fast organisms only.
A. Ten experiments. Original culture with emulsion containing rat-leprosy bacillus.	75	15	21	27	12
B. Five experiments. Sub-cultures from the tubes of experiments A showing acid-fast bacilli.	49	26	14	6	3
C. Two experiments. Sub-cultures from the tubes of experiments A showing a mixture of acid-fast bacilli and non-acid-fast organisms (examined daily for seven days).	8	8	..
D. Two experiments. Sub-cultures from the tubes from experiments A showing non-acid-fast rods and cocci (smearcd on the third day).	20	20

In most of the 21 tubes showing only acid-fast bacilli these organisms were very scanty and there was no suggestion of multiplication. In a few of these tubes there was a slight suggestion of multiplication but sub-cultures did not verify this.

Results of sub-culture.—Forty-nine sub-cultures were made from the tubes showing acid-fast bacilli. Of the sub-culture 14 showed scanty acid-fast bacilli, 6 showed acid-fast bacilli plus non-acid-fast organisms, and 3 showed only non-acid-fast organisms, and 26 showed no organisms of any kind.

Eight sub-cultures were made from four tubes showing both acid-fast and non-acid-fast organisms. From these sub-cultures smears were examined every day up to seven days. Smears from all the eight tubes showed a gradually increasing number of non-acid-fast organisms, only a few tubes showing the presence of

scanty acid-fast bacilli. Thus the non-acid-fast organisms found in the original tubes did not change into acid-fast bacilli on sub-culture into fresh media.

Twenty sub-cultures were made from two tubes showing non-acid-fast organisms only—in one case non-acid-fast rods and in the other case non-acid-fast cocci. All the 20 sub-cultures were examined on the third day after inoculation and in none of them did the organisms show any tendency to change into acid-fast forms.

FURTHER OBSERVATION ON STAINING PROPERTIES OF BACILLI.

To see whether there would be any change in the acid-fast character of the bacillus if kept in the minced tissue-media for a long time the following experiment was undertaken: A tube of this medium containing rat-leprosy bacilli was kept in the incubator and observed for two and a half months. Smears were taken at weekly intervals, stained and examined for the presence of acid-fast and non-acid-fast organisms. Scanty acid-fast bacilli were found till the ninth week, thereafter the smears were negative. No non-acid-fast organism was seen at any time. A sub-culture from the above tube was made after it had been in the incubator for four weeks. The sub-culture was observed for a period of one and a half months, smears being taken at weekly intervals. Acid-fast bacilli were found till the fifth week, thereafter the smears were negative. No non-acid-fast organism was seen at any time.

CONCLUSIONS.

Thus in the minced chick-tissue medium we were not able to verify the findings of McKinley and Verder, Soule, and Salle. We did not observe any multiplication of the acid-fast bacilli in this medium and in this respect our findings are similar to those of Holt. The bacilli were carried from one tube to another but there was no actual multiplication. The growth of the bacillus in alternating cycles of acid-fast and non-acid-fast phases was never seen.

From our experiments we have concluded that the non-acid-fast organisms encountered in minced tissue-media are simply contaminants and are not the non-acid-fast stage of the leprosy bacillus. From the nature of the work and of the procedures involved, particularly while working in a dusty city in the tropics, a certain percentage of contaminations is unavoidable.

SUMMARY.

1. In rat-tissue cultures and in minced chick-embryo medium no evidence of multiplication of rat-leprosy bacilli was obtained.

2. There was no evidence of change from acid-fast to non-acid-fast forms of the bacillus, and in the tissue cultures there was no difference in the staining properties between the bacilli in the tissue and the bacilli in the surrounding medium.

3. No change in the staining properties of the bacilli was observed under the following circumstances :—

(a) On death of the tissue culture.

(b) After seeding bacilli on dead tissues.

(c) After seeding bacilli on plasma and embryo extract without any tissue.

(d) After seeding and incubation in chick-embryo medium for ten weeks.

4. The only non-acid-fast organisms found in our preparations were apparently contaminants and they could not be converted into acid-fast bacilli by subculture in the presence of living tissue.

5. The finding of Salle that leprosy bacilli are acid-fast only in living tissues, and multiply in tissue cultures and chick-embryo medium in alternating acid-fast and non-acid-fast forms according to the conditions of the tissue, has not been confirmed.

6. The findings of McKinley and Verder, and Soule regarding the multiplication of leprosy bacilli in minced chick-tissue medium have not been confirmed.

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THE VIABILITY OF *LEISHMANIA DONOVANI* EXCRETED IN THE NASAL MUCUS IN INDIAN KALA-AZAR.

BY

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IN a previous communication (Shortt and Swaminath, 1935), following the work of Forkner and Zia (1934), we recorded the finding of *Leishmania donovani* in the nasal mucus of cases of Indian kala-azar. The material available to us for examination has been small in amount, but we have been able to record the presence of *L. donovani* in six out of fifteen cases examined.

As Forkner and Zia (1935) had already shown that in Chinese kala-azar the parasites found in the nasal mucus of cases were viable and capable of producing infections in hamsters, both when inoculated intraperitoneally and when instilled intranasally, it seemed probable that the same findings would be obtained in Indian kala-azar. To verify this point we inoculated three hamsters intraperitoneally with suspensions, in normal saline solution, of nasal mucus of cases of kala-azar, in the case of two hamsters introduced the suspension orally, and in another instilled it intranasally as well as introducing it orally. The details of our experiments are given below in tabular form.

It will be seen from the table that a positive result on intraperitoneal inoculation was obtained in one out of three cases. In one of these the duration of the experiment was too short for a satisfactory conclusion. In only one of the three cases where the material was introduced by the oral or nasal route was the microscopical finding positive in the material used, so that these experiments were not very satisfactory. In any case the fact is established that in Indian, as in Chinese, kala-azar the parasites excreted in the nasal secretions are viable and infective.

TABLE.
Showing details of experiments in which nasal secretion of kala-azar cases was used to infect hamsters.

Hamster (<i>Cricetus griseus</i>).	Infective material.	Route of administration.	Cause of termination of experiment.	Duration of experi- ment in days.	RESULT OF EXAMINATION.		Micro- scopical examination of material used as inoculum.	REMARKS.
					Micro- scopical.	Cultural.		
N. S. 2 ..	Nasal secretion from kala-azar case.	Intraperitoneally	Killed	200	Positive	Positive	Positive	Had two injections on alternate days from same case.
N. S. 4 ..	"	By mouth	"	211	Negative	Negative	"	..
N. S. 6 ..	"	Intraperitoneally	Died	43	"	Not done	Negative	Time insufficient for a satisfactory experiment.
N. S. 7 ..	"	"	Killed	192	"	Negative	"	..
N. S. 8 ..	"	By mouth	Died	106	"	Not done	"	Examination not satisfactory due to putrefaction.
N. S. 9 ..	"	Orally as well as intranasally.	"	86	"	"	"	"

The human case which gave the positive result is of interest in that it came from a district from which kala-azar has not previously been reported. The man, who is a native of Malabar, went to Coimbatore to work as a labourer, and commenced to get fever about a year after his arrival there. Neither of these districts had previously reported any case of kala-azar.

SUMMARY.

L. donovani occurring in the nasal secretion of cases of Indian kala-azar is viable and potentially virulent.

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STUDIES ON TYPHUS IN THE SIMLA HILLS.

Part VII.

ATTEMPTS TO ISOLATE A STRAIN OF XK TYPHUS FROM WILD RATS.

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MUCH evidence has already been obtained that a close relation exists between endemic typhus in men and a similar infection in rats. Strains of the virus from both sources have been examined and found to be identical. The rat-flea, *X. cheopis*, has been incriminated as the vector of the infection from rat to rat, and is accepted as the transmitter from rat to man.

In their studies on typhus in the Simla Hills Covell (1936b) and Covell and Mehta (1936) have demonstrated that in this endemic area also the rats are infected with a typhus virus, and two strains, one from the brains of wild rats, and the other from fleas collected from wild rats, have been isolated by them in guinea-pigs. Both strains conform to the X19 serological type of the disease.

In the Simla Hills two waves of typhus are usually found each year, each composed of a different form of the disease. Patients whose sera agglutinate *Proteus* X19 are seen mostly during winter; those giving agglutination with *Proteus* XK occur just after the monsoon, during the latter half of August, September, and October (Covell, 1936a).

Three types of typhus fever have been observed in India, when classified according to the Weil-Felix reaction. Boyd (1935) has shown that wherever they

occur, the majority of cases of typhus XK occurs just after the monsoon, typhus X2 and certain X19 types show a maximum incidence in December, while other X19 cases occur practically throughout the year. In Malaya, where the XK and X19 types are also prevalent, cases occur throughout the year. They have been designated 'rural' and 'urban' typhus, for irrespective of their seasonal incidence the XK type is invariably contracted in rural, and the X19 type in urban, areas. The flea, *X. cheopis*, is accepted as the vector of the urban form and a larval trombiculid mite is suspected to be the vector of the rural form, the rat being the suspected reservoir of both forms of infection (Lewthwaite and Savor, 1934a).

The distinct seasonal variation of the two forms of typhus in this area offers, we think, exceptional advantages for investigating the insect vector of the two forms.

An entomological survey of the ectoparasites of wild rats was begun in this area in 1935. This has now been completed. With a view to testing the rôle of the different ectoparasites found on wild rats with the two strains of typhus which occur in this area, an endeavour was made to isolate a strain of the XK form from wild rats, on the assumption that here, as in Malaya, they are the reservoir of both forms of typhus.

The Weil-Felix reaction with the sera of wild rats, trapped in Kasauli and the surrounding villages, was therefore systematically carried out from May last year. A total of 1,212 sera was examined up to the end of February this year, and the results are appended in tabular form. It will be seen that in September there is a rise in the number of sera giving agglutination with *Proteus* X19 in the 1 in 50 dilution. In October, November, and December there is a rise in the number agglutinating with *Proteus* XK in the 1 in 125 dilution. With the exception of two sera, all the others which showed agglutination in a higher dilution were hæmolyssed. Being already in possession of strains of the X19 type in guinea-pigs no further use was made of those rats whose sera agglutinated *Proteus* X19 in a dilution of 1 in 50 or over. Of the two rats whose sera agglutinated *Proteus* XK in a dilution of 1 in 250, one unfortunately died shortly after its blood was taken. The other, as well as one whose serum was hæmolyssed, was investigated further. Rats whose sera showed some agglutination in the 1 in 125 dilution were not investigated, as this was considered too low a titre to be of much significance when nearly 84 per cent of all rats show a total or standard agglutination in the 1 in 50 dilution with *Proteus* XK.

Rat 2626.—Serum hæmolyssed. W. F. reaction = X19—trace 1 in 1,000. XK trace 1 in 1,500. This rat was selected as it was trapped in the same house as rat 2625 whose serum also agglutinated up to 1 in 125 XK. Both rats were heavily infested with mites, *L. bacoti*. The mites from rat 2626 were emulsified in saline and injected into two guinea-pigs intraperitoneally. Neither pig showed any reaction. The mites from rat 2625 were similarly injected into another guinea-pig with negative results.

Nineteen *C. simla* were allowed to feed on rat 2626 for seven days, on the 8th day they were emulsified and injected intraperitoneally into a guinea-pig. No reaction.

A nymph of *Rhipicephalus* was found on this rat. After it had fed to repletion, it was removed and placed in a test-tube till the adult emerged. The tick was emulsified and injected into a guinea-pig. No reaction.

The brain and spleen of this rat were emulsified separately and injected into two guinea-pigs. Neither showed any reaction.

TABLE.
Results of *Weil-Felix* reactions on wild rats.

Month.	Total W.R.'s	DILUTIONS.										REMARKS.
		X10.					XK.					
		1-25.	1-50.	1-125.	1-250.	1-25.	1-50.	1-125.	1-250.			
		6	2	1	0	6	65	5	3*			
May ..	79	6	2	1	0	6	65	5	3*	*2 sera hemolysed.		
June ..	156	10	0	0	1	11	124	7	1*	*1-25 dilution not done in 13.		
July ..	145	9	0	0	0	16	114	3	0	*1-25 dilution not done in 12.		
August ..	196	7	2	1*	0	21	163	3	0	*1-25 dilution not done in 8. 1 negative.		
September ..	181	20	11	3	3*	7	164	3	3*	*Hemolysed sera. 1-25 dilution not done in 4.		
October ..	133	6	5	1	1*	7	109	16	1*	*Hemolysed serum.		
November ..	78	7	0	1	0	0	62	15	0	*1-25 dilution not done in 1.		
December ..	101	5	2	3	1	2	86	12	1			
January ..	90	7	1	1	0	1	85	3	0	*1-25 dilution not done in 1.		
February ..	53	2	3	0	0	0	47	4	0	2 not done in 1-25 dilution.		
TOTALS ..	1,212	79	26	11	6	71	1,019	71	9	1-25 not done in 41. Negative 1.		

Rat 3421.—W. F. reaction = X19—1 in 25 trace. XK 1 in 250 trace. Ten *X. cheopis* found on this rat were emulsified in saline and injected into guinea-pig 3421F. The highest temperature recorded was 102.8°F. on the 5th day. It was killed on the 13th day after inoculation. *Post-mortem*.—Spleen slightly enlarged and congested. Testes normal. Spleen and brain emulsion of this animal were injected into guinea-pigs 3421F2 and 3421F3. Guinea-pig 3421F2 showed no reaction. F3 had fever. The temperature rose to 103.8°F. on the 5th day, but it was also noted that this animal had an abscess on the neck. It was killed and passaged to guinea-pig 3421F4 on the 12th day. This animal showed no reaction.

The brain of rat 3421 was emulsified and injected into guinea-pig 3421B intraperitoneally. This animal showed a temperature of 103°F. on the 6th day. It was killed on the 11th day. *Post-mortem*.—Nothing abnormal seen. The brain and spleen was emulsified and injected into 3421B1. No reaction noted in this animal.

To determine whether any mutation of the virus would occur in its passage through an arthropod other than the flea, as well as to investigate their rôle in the transmission of X19 typhus, larvæ and nymphs of *R. sanguineus* as well as mites *L. bacoti* bred in the laboratory were fed on guinea-pigs and rats infected with the laboratory strain of typhus X19. After varying periods they were emulsified and injected into guinea-pigs.

- (1) Ten nymphs of *R. sanguineus* were fed on an infected guinea-pig. Five to seven days after engorgement they were emulsified and injected into guinea-pig R. N. 1. No reaction, febrile or otherwise.
- (2) Twelve larval ticks were fed on the same infected animal as above. Four to six days after engorgement they were emulsified and injected into guinea-pig R. L. 2. No reaction.
- (3) Seven larval ticks were fed on white rat 176. Six to seven days after they had engorged they were emulsified and injected into guinea-pig R. L. 3. No reaction.
- (4) Fourteen larval ticks were fed on wild rat 2862, which was infected in the laboratory with the X19 strain. One to seven days after engorgement they were emulsified and injected into guinea-pig R. L. 4. No reaction.
- (5) Ten larval ticks were fed on a white rat 177. Five to eight days after engorgement they were emulsified and injected into guinea-pig R. L. 5. No reaction.
- (6) Twenty-five mites, *L. bacoti*, fed on infected guinea-pig 188F1, were emulsified and injected into a guinea-pig eight days after feeding. No reaction.

It might here be mentioned that a batch of fleas *C. simla* fed on an infected white rat under similar conditions, and later emulsified and injected into a guinea-pig, produced a scrotal reaction and fever typical of typhus X19.

At the time when cases of typhus, XK type, were occurring in the human population, to determine what part, if any, fleas played in the transmission of the disease, fleas collected from wild rats were emulsified and injected into guinea-pigs.

- (1) 28-8-36. Wild rat 2960. W. F. reaction = X19—negative. XK 1 in 50 total. Wild rat 2965. W. F. reaction = X19—negative. XK 1 in 50 total minus. Seven fleas collected from these two rats were emulsified and injected intraperitoneally into guinea-pig 60-65F. This animal showed a drop in temperature on the 14th day to 99.6°F. The highest temperature recorded was 102°F. It was killed and passaged to guinea-pigs 60-65F1 and F2. Neither guinea-pig showed any reaction.
- (2) 28-8-36. Wild rat 2963. W. F. reaction = X19—negative. XK 1 in 50 total. Wild rat 2964. W. F. reaction = X19—negative. XK 1 in 50 total. Nine fleas recovered from these rats were emulsified and injected into guinea-pig 63-64F. This animal had a temperature of 103°F. on the 13th day. It was killed the next day and spleen emulsion passaged to guinea-pigs 63-64F1 and F2. F1 showed a considerable loss of weight by the 8th day. It was killed and passaged to guinea-pigs 63-64F3 and F4. Both these animals died without showing any symptoms, one on the 8th and the other on the 17th day.

Guinea-pig 63-64F2 showed a typical scrotal reaction and fever on the 7th day. It was killed and passaged to guinea-pigs 63-64F2A and F2B. Both animals showed a scrotal reaction and fever on the 7th day.

- (3) 15-10-36. Wild rat 3225. W. F. reaction X19—negative. XK 1 in 125 trace. Wild rat 3228. W. F. reaction X19—negative. XK 1 in 125 trace.

Eleven fleas recovered from these rats were emulsified and injected into guinea-pig F. W. 2. This animal showed a rise in temperature to 103.2°F. on the 8th day. Owing to scarcity of guinea-pigs this animal was not passaged.

Twenty laboratory-bred *X. cheopis* fed on these two rats were emulsified and injected into guinea-pig F. W. C2 on 21-10-36. This animal showed fever and a typical scrotal reaction on the 8th day.

- (4) 18-10-36. Wild rat 3251. W. F. reaction = X19—negative. XK 1 in 50 total. Wild rat 3252. W. F. reaction = X19—negative. XK 1 in 50 total.

Eighteen fleas recovered from these rats were emulsified and injected into guinea-pig F. W. 3. This animal showed a marked scrotal reaction on the 7th day with a temperature of 104°F.

- (5) 19-10-36. Wild rat 3256. W. F. reaction = X19—negative. XK 1 in 50 total. Wild rat 3257. W. F. reaction = X19—negative. XK 1 in 25 std: 13 fleas (11 *X. cheopis* and 2 *C. simla*) found on these rats were emulsified and injected into guinea-pig F. W. 4. This animal showed a temperature of 103.3°F. on the 10th day. It was not passaged owing to lack of animals.

- (6) 21-10-36. Wild rat 3275. W. F. reaction = X19 negative. XK 1 in 50 total. Thirty fleas collected from this rat were emulsified and injected into guinea-pig F. W. 5. This animal showed fever and a typical scrotal reaction on the 9th day.

DISCUSSION.

Efforts to isolate a strain of typhus XK from wild rats selected on the results of the Weil-Felix reaction to detect naturally infected animals were not successful. Covell (1936*b*) obtained a pure strain of typhus X19 when the pooled brains of three rats, giving Weil-Felix reactions both to XK and X19, were injected into guinea-pigs. This strain is now in its 84th passage, and has remained unchanged. Lewthwaite and Savor were successful in isolating two strains of typhus XK from wild rats.

Attempts to transmit the X19 virus by the agency of ticks, *R. sanguineus*, and mites, *L. bacoti*, were not successful. There was also no evidence of any mutation of the virus having occurred in these arthropods.

In the last series of experiments it will be seen that on three occasions out of six, when fleas collected from wild rats were emulsified and injected intraperitoneally into guinea-pigs, a scrotal reaction and fever indicative of typhus X19 were obtained. In the third and fifth experiments of this series, it is very probable that if the passages were persisted with, scrotal reactions would also have been demonstrated. In the third experiment, clean laboratory-bred fleas which were fed on the rats in question acquired infections, as evidenced by the scrotal reactions and fever when they were emulsified and injected into a guinea-pig.

It is unfortunate that it was not possible to space these tests regularly during the post-monsoon period. This was due to the demand for guinea-pigs to maintain four strains of human typhus (XK type) being so great that no animals could be spared at the critical time. There is nevertheless enough evidence that infected rats and rat-fleas were much in evidence at the time. That the reactions were due to infections with the virus of typhus X19 may be assumed from the fact that the

reactions were caused by the injection of emulsions of fleas which preclude the scrotal reactions being due to other causes, such as the injection of normal brain tissue, rat-bite fever, etc. The duration of the fever and the scrotal reaction were also in keeping with infections of typhus X19.

The sera of the rats from which the fleas were collected gave no agglutination with *Proteus* X19. Two of them gave agglutination with *Proteus* XK in a dilution of 1 in 125, and of the remainder all but one gave a total agglutination in the 1 in 50 dilution. Harvey (1936) summarizing the work of Covell and Mehta on the rôle of the rat-flea in the transmission of typhus in the Simla Hills wants to know why the virus isolated from rats and fleas produces agglutinins for OX19 only, while the serum of the rats show agglutinins for OXK. These results cast a doubt on the value of the Weil-Felix reaction in detecting infections in wild rats, for it is unlikely that the fleas had acquired their infections from other rats or another species of rodent, as in one experiment at least it was demonstrated that the rats themselves were infected.

The isolation of strains of the X19 form of typhus from fleas or rats which gave no significant Weil-Felix reactions has been the experience of other workers in India, Malaya, in certain European countries and America. It thus seems unlikely that fleas are responsible for the transmission of the XK form of typhus here. The indefinite results obtained by Lewthwaite and Savoor (1934b) in attempts to transmit typhus (XK type) by fleas under laboratory conditions also support this view.

That no cases of typhus X19 occur here during the post-monsoon period when fleas *X. cheopis* are in their maximum numbers on the rats, and have been shown to be infected, is remarkable if the human and murine viruses are identical and the flea is the transmitter from rat to man. Brumpt (1932), comparing the incidence of the disease in man and the infection among the rat population, raises a doubt regarding the identity of the two viruses. Cases of the X19 form of typhus, however, occur in this area during winter, when the number of arthropods is generally very scanty. As the flea most probably does not transmit the infection by its bite, perhaps it is some association between man and rat, other than by the agency of the flea, which results in an infection.

That the XK type has been designated 'rural' in Malaya, where a regional distinction exists between this form and the X19, is suggestive that a jungle rodent is the probable reservoir of the infection. Other than rats, the only rodent uninvestigated in India in connection with its rôle as a reservoir of typhus was the squirrel, *Sciurus palmaris* (Shortt and D'Silva, 1936). They showed that there was a difference in the Weil-Felix reactions with the sera of squirrels captured in Kasauli compared with others from the plains.

Megaw (1925) suggested the wild hare as the probable reservoir of infection. Inquiries early this year have elicited the fact that in the cantonment of Sabathu, which incidentally produced the largest number of cases of XK typhus last season, hares are very common in the scrub jungle which encroaches close to many of the barracks and dwelling houses. Under such conditions no special trip into the jungle need be made for anyone to expose himself to the bites of ectoparasites from jungle rodents. It is hoped to investigate the rôle of these rodents in the near future.

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STUDIES ON TYPHUS IN THE SIMLA HILLS.

Part VIII.

ECTOPARASITES OF RATS AND SHREWS WITH SPECIAL REFERENCE TO THEIR POSSIBLE RÔLE IN THE TRANSMISSION OF TYPHUS.

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It has been pointed out by Covell (1936) that in the Simla Hills, as in Malaya, there exist two types of typhus fever, the serum of one agglutinates strains of *Proteus* XK and not X19, whilst that of the other gives the opposite reaction. Cases of the XK type of typhus are reported in this area during the latter half of August and September, and cases of the X19 type during mid-winter and early spring. It is probable that the seasonal incidence of the two types of typhus in this area is dependent on the prevalence of different arthropod vectors on the possible rodent reservoirs of infection at these particular periods.

The incidence of the XK type of the disease, according to the Malayan workers (Anigstein, 1933; and other workers), is closely connected with life in a rural environment, while the X19 type is found among people living and working in urban areas. It seems that the conditions existing in Malaya have a close parallel in the cantonment areas of Kasauli and Sabathu in the Simla Hills, where both these forms of typhus have been recorded. Typically urban conditions are found in the bazaar sections of these cantonments, where insanitary conditions to a greater or lesser degree prevail, due mostly to overcrowding. Rural or semi-rural conditions are represented in the military and civil areas where the barracks and dwelling houses are scattered over open areas with, in many cases, patches of scrub jungle interspersed between such buildings. It will thus be seen that in a circumscribed area, conditions suited to the transmission of both forms of typhus exist.

This report is based on investigations of the ectoparasitic fauna present on members of the families *Muridæ* and *Soricidæ*. The former includes a variety of rats and the latter the shrews or musk-rats. The work was considered important in view of the fact that rats are the accepted reservoir of typhus in other countries, and have been shown to be harbouring a virus identical with that obtained from human sources. Moreover, on account of the habits of these animals to live in diverse situations, such as in houses, in the open areas where they burrow, and in trees where they build their nests amongst the branches, it was expected that their ectoparasites would be fairly representative of both the rural and urban fauna, thus affording the most likely clues to the solution of the arthropod vectors of the two forms of typhus.

RATS AND SHREWS TRAPPED AT KASAU LI AND SABATHU.

A total of 2,451 rodents belonging to the families *Muridæ** and *Soricidæ** was trapped during the year 1935-36. The majority of these belonged to the common dark or brown variety of rats, *Rattus rattus*. Considerable variation exists in this genus. A large number of *decumanus* rats was also captured. A fuller account of the races of these rats will be given in a separate paper. The other genera of the family *Muridæ* trapped include the Indian mole-rat *Nesocia bengalensis*, the short-tailed mole-rat *Nesocia hardwickei*, the Indian bush-rat *Golunda ellioti*, the common house-mouse *Mus musculus*, and the Persian house-mouse *Mus bactrianus*. Of the family *Soricidæ* several members of the genus *Suncus* (*Crocidura*) *cæruleus tytleri* (the Himalayan grey musk-shrew) were captured from Kasauli and Sabathu.

Table I gives the monthly distribution of rats and shrews during the year 1935-36 :—

TABLE I.

Month.	<i>Rattus rattus</i> .	<i>Mus musculus</i> .	<i>Mus bactrianus</i> .	<i>Nesocia</i> .	<i>Golunda ellioti</i> .	<i>Suncus cæruleus tytleri</i> .
June 1935 ..	137	9	1	0	1	0
July 1935 ..	311	10	0	1 (<i>hardwickei</i>)	0	0
August 1935 ..	254	0	0	1 (<i>bengalensis</i>)	0	2
September 1935 ..	301	0	0	0	0	0
October 1935 ..	374	0	0	1 (<i>bengalensis</i>)	0	4
November 1935 ..	282	0	1	1 (<i>bengalensis</i>)	0	3

*A general account of Indian *Muridæ* and *Soricidæ* is given by Blandford (1891) and Lindsay (1929), respectively.

TABLE I—conclld.

Month.	<i>Rattus rattus.</i>	<i>Mus musculus.</i>	<i>Mus bactrianus.</i>	<i>Nesocia.</i>	<i>Golunda ellioti.</i>	<i>Suncus caeruleus tytlteri.</i>
December 1935 ..	175	0	0	0	0	3
January 1936 ..	158	3	0	0	0	1
February 1936 ..	144	0	0	0	0	1
March 1936 ..	93	3	0	0	0	1
April 1936 ..	75	1	0	0	0	0
May 1936 ..	98	0	0	0	0	0

It is evident from the above that the number of mole-rats and bush-rats is exceedingly small compared with that of the common rat *Rattus rattus*. The traps were laid only in dwelling houses and shops, both in the urban and semi-rural areas, hence this was to be expected, as the former would seek refuge in such places only under exceptional circumstances. The usual habitat of these rodents is in open country, both cultivated and waste land. In a single instance a mole-rat was trapped outside its burrow and a bush-rat was captured in the vicinity of the Director's bungalow in the Pasteur Institute estate.

Strickland (1927) has given a long list of rodents found in various parts of India and he remarks that both the distribution of *Mus rattus*, *Mus decumanus*, or *Mus bactrianus*, and other related species, and their relation to forest, cultivation, and man, are consistent with the incidence of the reported cases of 'pseudo-typhus'. This is also true in the case of mole- and bush-rats, the latter living in the jungle and nesting in or under bushes.

ECTOPARASITES.

Amongst the arthropods occurring as ectoparasites on rats and shrews in the Simla Hills are included the fleas of the genera *Xenopsylla*, *Ceratophyllus*, *Otenocephalus*, and *Leptopsylla*. The principal genera of mites observed are *Liponyssus*, *Dermanyssus*, *Echinolaelaps*, and larval forms of *Trombicula*. The ticks are represented by *Hyalomma*, *Rhipicephalus*, *Hæmaphysalis*, and *Ixodes*. The rat-lice belong to the genus *Polyplax*. The details regarding their seasonal distribution are given below :—

(1) *Fleas*.—It has been observed that the majority of fleas on rats in the Simla Hills are *Xenopsylla cheopis*. They are found all the year round but are conspicuously abundant during the months of September and October. During the winter when it is very cold in the hills they show a marked decrease in numbers. It has been stated (Sharif, 1930) that in certain hill-stations *Xenopsylla cheopis* is the only flea found on rats. In the Simla Hills, however, this is not the case, there being four distinct genera present on rats, *X. cheopis* being undoubtedly the predominating

species, particularly during the summer. *Ceratophyllus simla* is the other flea frequently noticed on rats. During the months of October and November fleas of the genus *Ceratophyllus* are in their maximum numbers. Later, with the advent of the cold weather their numbers though not so pronounced are appreciably larger and during February and March they actually outnumber the other rat-flea *Xenopsylla cheopis*.

The dog- and cat-fleas, *Ctenocephalus canis* Curtis and *C. felis* Bouché, are also found on rats but in exceedingly small numbers. It appears they are only accidental ectoparasites of rodents. Another flea, *Leptopsylla segnis* Schönh., has also been observed on rats during the post-monsoon period and extending to early spring.

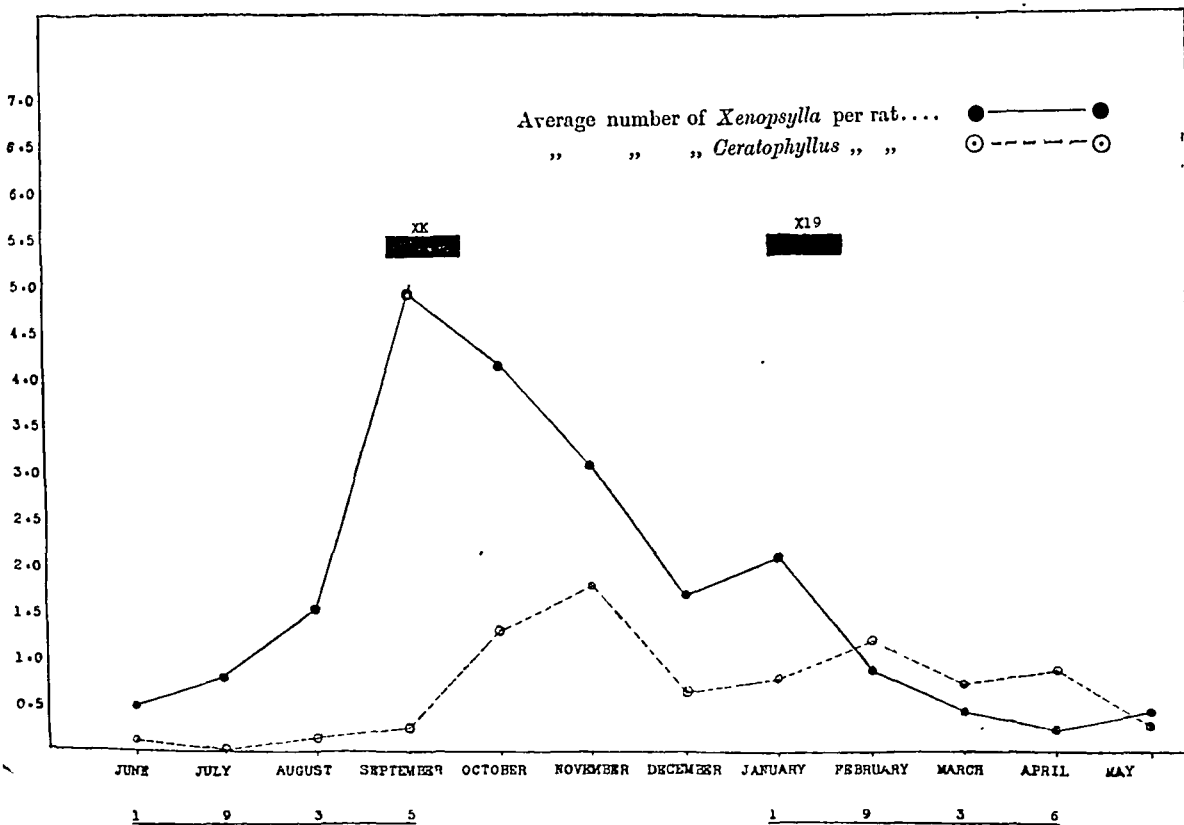
The actual distribution of fleas on rats has been given in Table II:—

TABLE II.

Month.	Total rats trapped.	FLEAS.			
		<i>Xenopsylla.</i>	<i>Ceratophyllus.</i>	<i>Ctenocephalus.</i>	<i>Leptopsylla.</i>
June 1935 ..	148	74	17	0	0
July 1935 ..	322	268	14	2	0
August 1935 ..	257	404	35	0	1
September 1935 ..	301	1,502	77	3	1
October 1935 ..	380	1,596	528	0	0
November 1935 ..	287	896	516	1	5
December 1935 ..	178	301	113	0	0
January 1936 ..	162	346	128	1	11
February 1936 ..	145	131	176	0	4
March 1936 ..	97	41	72	0	7
April 1936 ..	76	18	67	0	0
May 1936 ..	98	42	30	0	0

Chart 1 also shows the comparative seasonal incidence of *Xenopsylla cheopis* and *Ceratophyllus* sp. in relation to seasonal prevalence of the two serological types of typhus:—

CHART 1.



During the time when typhus fever of the XK type is occurring in the human population fleas of the species *Xenopsylla cheopis* are found on rats in their maximum numbers. This incidence of fleas would evidently lead one to suspect them as possible vectors of this disease.

Mites.—There are four distinct genera of mites found on the common rat, *Rattus rattus*, and other allied members of the family *Muridae*. These include the common rat-mite *Liponyssus bacoti* Hirst, *Dermanyssus* sp., *Echinolaelaps echidninus* Berlese, and larvæ of the genus *Trombicula*.

Table III indicates the relative distribution of various genera of mites on rats during the year 1935-36 :—

TABLE III.

Month.	<i>Liponyssus bacoti.</i>	<i>Dermanyssus</i> sp.	<i>Echinolaelaps echidninus.</i>	<i>Trombiculæ.</i>				
				<i>indica.</i>	<i>oude-mansi.</i>	<i>deliensis.</i>	n. sp.	<i>acuseu-tellaris.</i>
June 1935 ..	139	0	15	67	1	34	34	0
July 1935 ..	577	3	20	206	13	89	205	0
August 1935 ..	271	10	3	174	438	342	80	0
September 1935 ..	139	2	0	376	194	153	15	0
October 1935 ..	257	8	0	611	198	116	1,173	0
November 1935 ..	162	3	5	3	45	228	1,426	0
December 1935 ..	55	0	1	0	0	232	828	11
January 1936 ..	6	1	0	0	0	100	926	0
February 1936 ..	45	1	0	0	0	41	375	0
March 1936 ..	8	0	2	0	10	41	577	0
April 1936 ..	2	0	17	0	0	43	327	0
May 1936 ..	69	0	0	0	17	36	51	0

The tropical rat-mite, *Liponyssus bacoti* Hirst, is commonly found on rats all the year round in all stages of development except during the cold months ranging from January to March. It is a very prolific breeder, the length of its life-cycle being very short and confined to about a week during the summer months. Further details of its life-history as observed in the laboratory will form the subject of a separate paper.

Dermanyssus sp. is not commonly found on rats but it is only an accidental parasite. This observation is amply substantiated by the data given above. Strickland (*loc. cit.*) has quoted Hirst to show that *Dermanyssus muris* and *Dermanyssus gallinae* occur on rats all over the world. The latter has been recorded to bite man.

Another mite, *Echinolaelaps echidninus* Berlese, which has been described by Ewing (1929) as a common arachnid on the domestic species of rats in various parts of the world, has also been found on rats in Kasauli and Sabathu, although in very small numbers. Strickland (*loc. cit.*) has mentioned that *Laelaps echidninus* has been recorded from Bombay and Calcutta infesting rats and these mites were identified by Hirst. An allied species *Laelaps nuttalli* has also been described by Hirst (1915) from Calcutta. This specimen was found on the Indian mole-rat, *Nesokia bengalensis*, and on *Rattus rattus*.

Larval mites of the genus *Trombicula* have been quite frequently found on rats and have been located on the hairless parts within the ears and around the rims of the eyes. In a very few cases they have also been detected attached around the anus. Five species of larval *Trombiculae* have been noticed on rats captured from Kasauli and Sabathu, namely *Trombicula indica* (*muris*) Walch, *T. oudemansi*, *T. deliensis*, *T. acuscutellaris*, and another probably a new species. Of all these *T. deliensis*, *T. acuscutellaris*, and the new species (to be described later) have been recorded for the first time by me in India while *T. indica* (*muris*) and *T. oudemansi* have been previously reported by Hirst (*loc. cit.*) to be parasites of *Nesokia bengalensis* and *Rattus rattus* from Calcutta. He has, however, preferred to call them *Schöngastia indica* and *Schöngastiella bengalensis* respectively and has labelled them as new species. It is believed that these two species of *Trombicula* do not attack man but are very common ectoparasites of rats.

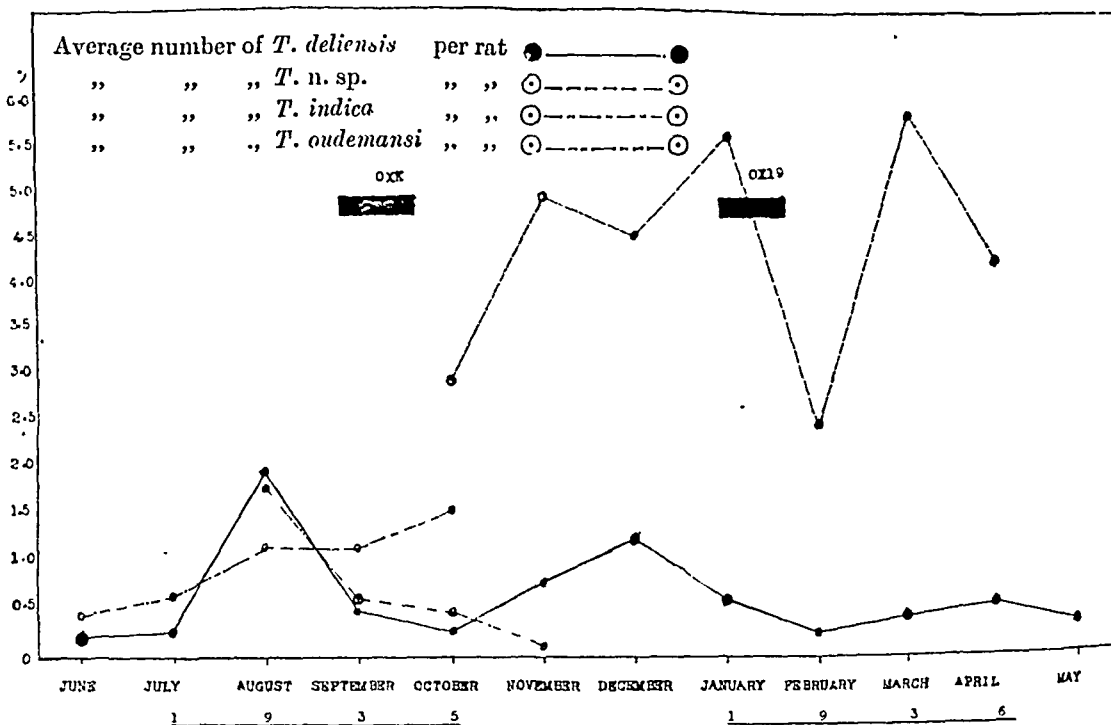
It has been shown above that *Trombicula indica* (*muris*) is the predominating species during the summer and continues to remain so till October, after which it becomes very scarce. The other allied species *T. oudemansi*, however, does not appear in large numbers till August and then disappears almost entirely with the advent of cold weather. The most important species to be considered is *Trombicula deliensis* which has been suspected to be the vector of typhus in Deli. It is cosmopolitan in distribution and has been observed on rats throughout the year, though in varying numbers. It appears that this species can stand the rigours of cold very well and in this respect its only other equal is the new species of *Trombicula* found in the Simla Hills. Judging from the morphological characters of the two species they appear to be almost identical and could only be separated by minor details in chaetotaxy. A systematic account of the various species of *Trombicula* recorded from this area has been reserved for publication separately. The only other *trombiculid* larva found on rats and known to bite man is *T. acuscutellaris*. Only in a few instances has this species been reported and that too in the month of December. This is also recorded for the first time on rats in India.

Details regarding the prevalence of these mite larvæ during the year 1935-36 have been embodied in Chart 2.

Ticks.—Of all the acarina which attack man, ticks have been very largely incriminated in the transmission of disease. A careful survey was, therefore, made and ticks present on rats were studied. Four genera of ticks, namely *Hyalomma*,

Rhipicephalus, *Hæmaphysalis*, and *Ixodes*, mentioned in order of their prevalence were observed on rats. *Hyalomma* larvæ and nymphs have been found in greatest abundance. It appears that the adults engorge mainly on higher mammals. I have isolated them from goats in New Delhi and from sheep at Kasauli. Patton and Cragg (1913), in an examination of 3,000 calves used for vaccination purposes at the King Institute of Preventive Medicine, Guindy, Madras, found the nymphs of *Hyalomma* twenty-four times, while the adults were present on almost every calf brought to the institute. They also recorded the presence of these adult ticks on horses, dogs and the south Indian hare *Lepus nigricollis*. These workers produced experimental evidence to show that the larvæ of *Hyalomma* engorge only on lower mammals such as guinea-pigs and rats and refused to feed on calf, goat, pig, dog,

CHART 2.



cat, etc. It would, therefore, be wise not to ignore this point when making any generalizations on the transmission of typhus through the agency of this tick.

It will be abundantly clear from the data given below (Table IV) that *Hyalomma* ticks are present on rodents in appreciably large numbers during September and slowly disappear with the advent of cold weather in the following months. In the months of May and June when the temperature is high and the humidity is low these ticks especially their immature stages are rarely seen. The biotic potential is raised with the coming monsoon rains with the result that a larger number of individuals are produced and can survive through the more favourable environmental conditions. This is really why we have the largest numbers of individuals

on record during September as at this period the optimum conditions exist for their survival.

Hyalomma aegyptium is the species most commonly found on cattle, particularly sheep and goats. Sharif (1928) has remarked that this species generally attacks cattle, horse, camel, goat, donkey, and occasionally dog and bear.

The following details given in Table IV show the distribution of the ticks on rats during the various months :—

TABLE IV.

Month.	<i>Hyalomma.</i>	<i>Rhipicephalus.</i>	<i>Hæmaphysalis.</i>	<i>Ixodes.</i>
June 1935 ..	5	40	0	0
July 1935 ..	87	22	0	1
August 1935 ..	80	24	0	3
September 1935 ..	246	35	12	0
October 1935 ..	14	33	12	0
November 1935 ..	7	1	16	1
December 1935 ..	5	0	3	0
January 1936 ..	1	0	0	0
February 1936 ..	3	0	0	0
March 1936 ..	23	1	2	0
April 1936 ..	1	65	1	0
May 1936 ..	2	0	0	0

There are two species of *Rhipicephalus* found on rats and shrews in this area, namely *R. sanguineus* and *R. hæmaphysaloides*. The former is much more common and only the larvæ and nymphs are frequently noticed. The other species is very scarce which is explained by the fact that it is found mainly in waste land and is picked up by the rodents during their sojourn in such areas.

Ticks of the genera *Hæmaphysalis* and *Ixodes* are only rarely seen on rats and in this connection it may be mentioned that larvæ and nymphs of the former are chiefly present on rats while the adults of *Ixodes* sp. have been observed. The immature stages of *Hæmaphysalis* manifest themselves during the months of September, October, and November. The distribution of *Ixodes* sp. is very irregular.

Lice.—The lice on rats collected at Kasauli and Sabathu belong to a single genus *Polyplax* and the common species recorded is *Polyplax spinulosa*. They are

nearly equally distributed during the summer and winter except during the months of May, June, and July when the temperatures are comparatively high and the humidity is very low.

Data regarding their numerical strength during the year 1935-36 are given in Table V :—

TABLE V.

Month.	Number of lice.	Month.	Number of lice.
1935.		1936.	
June ..	42	January ..	689
July ..	76	February ..	514
August ..	333	March ..	369
September ..	593	April ..	175
October ..	927	May ..	92
November ..	659		
December ..	566		

It is very difficult to enumerate accurately the presence of lice on their host due to the fact that they cling to the hair very closely. To isolate and disentangle each specimen from the living animal is a very lengthy process and it is not unlikely that one might miss many of them. It is, therefore, possible that the figures given above would only give an approximate idea of their numerical strength.

POSSIBLE RÔLE OF ECTOPARASITES IN TYPHUS TRANSMISSION.

Cases of the XK type of typhus have been reported in the Simla Hills during the past years in the months of August, September, and October. At this time nearly all the ectoparasites have a high biotic potential, the physical conditions of the environment being very favourable for their survival. Consequently it is hardly possible to incriminate any particular arthropod on a basis of numerical strength alone. It would perhaps be useful to discuss the factors involved.

Although other genera of fleas are present on rats it is only *Xenopsylla cheopis* which shows a marked seasonal incidence corresponding with the prevalence of this disease. Judging from the numerical strength of this insect one would expect a kind of rural plague if it could transmit the disease from rat to man but this does not seem to occur in nature. Experimental attempts to transmit the virus of rural typhus with *X. cheopis* carried out by Lewthwaite and Savor (1934) were unsuccessful. Further work on these lines is necessary before the possibility that these insects may be concerned is dismissed.

The possible relationship of fleas as vectors of the urban form of typhus has been thoroughly investigated by Lewthwaite and Savor (loc. cit.) who showed that the flea *X. cheopis* can acquire a virulent infection under experimental conditions and their findings leave little doubt that, in nature, the rat-flea is a vector of urban typhus from rat to rat and from rat to man. These findings have been recently confirmed by Covell and Mehta (1936) and further work (Smith and Mehta, 1937) has shown that the other rat-flea, *Ceratophyllus simla*, can also harbour the virus of the X19 type of typhus. It is possible that similar results may also be obtained with the other fleas found on rats.

The rôle of mites in the transmission of tropical typhus has received considerable attention during the last ten years. This importance is due to the fact that some of the mites occurring as ectoparasites on rats and shrews also bite man. It has been shown above that the tropical rat-mite, *Liponyssus bacoti* Hirst, has been commonly observed on rats during the time when cases of the XK type of typhus occurred in the Simla Hills. The fact that this arthropod often attacks man and causes dermatitis would certainly offer possibilities that it may be a vector of this disease. In fact it has already been shown by Shelmire and Dove (1932) that the Texas strain of endemic typhus was experimentally transmitted through the bites of *Liponyssus bacoti* Hirst, in laboratory animals. They have also emphasized that the frequency with which this mite takes the blood from an animal, coupled with the short periods required for their growth and the number of different animals they are apt to feed upon, favours the transmission of endemic typhus by these mites from rat to rat and from rat to man. This work has not received any confirmation from any other source. In attempts to infect this mite with typhus of the X19 type it was found (Smith and Mehta, loc. cit.) that *Liponyssus bacoti* does not take up and develop the virus. Further experimental work is in progress to determine whether this mite plays any part as a carrier of this disease in the Simla Hills. Strickland (loc. cit.) has suggested that the rats and *Liponyssus bacoti* be investigated in relation to typhus transmission. The fact that this mite readily attacks man and rat and is a very prolific breeder, would make it a very efficient carrier. If it could harbour the virus of typhus one would naturally expect an epidemic of typhus occurring during the post-monsoon period. But this is not so and the conclusion would be that though noxious to man in other ways this arthropod probably does not play any part as a vector of this disease here.

Of all the other mites found on rats only the larval *Trombiculae* need attention. It has been proved that *Trombicula akamushi* is the vector of the Japanese river-fever or Tsutsugamushi disease and an allied species *Trombicula deliensis* has been incriminated in the transmission of tropical typhus in Deli. Gater (1930) has suggested that *Trombicula akamushi* and *T. deliensis* are merely forms of the same species and has concluded that the vector of tropical typhus may prove to be the same as for the Japanese river-fever.

There are five genera of larval *Trombiculae* present on rats in the Simla Hills and of all these *T. deliensis* and *T. acuscutellaris* deserve special mention since they are reported to attack both rat and man. The mites *T. deliensis* are found on rats all the year round and are particularly abundant during the season when cases of the XK type of typhus occur at Kasauli and Sahathu. Moreover, it is highly probable that rodents are attacked by larval *Trombiculids* when they burrow in the open country since the latter abound in loose earth and such other places. This is

supported by the fact that these mites have been captured by me on the mole-rats trapped at Kasauli under semi-rural conditions. It is, therefore, emphasized that the possibility of *Trombicula deliensis* acting as a vector of rural typhus should not be ignored. The other species, *Trombicula acuscutellaris*, which has also been recorded to bite man in other countries, is found only in the month of December and that too in very small numbers. It is highly probable that this species has no relation to the transmission of this disease.

The parasitid mite, *Echinolaelaps echidninus*, which is also found on rats is not known to attack man (Ewing, *loc. cit.*). The chicken-mite, *Dermanyssus* sp., reported on rats appears to be only an accidental parasite and it is not known whether it attacks man as readily as the tropical rat-mite, *Liponyssus bacoti*.

Ticks have been strongly suspected as vectors of typhus in India. According to Megaw (1924) the evidence available in the Kumaon Hills suggests that the typhus-like fever may be tick-borne and that the reservoir is a jungle-living animal. He considers that it is most likely the hare or the palm-squirrel which is the reservoir and the vector *Hyalomma aegyptium* or *Rhipicephalus sanguineus*. This view is supported by Strickland (*loc. cit.*).

In the present survey of the ectoparasites it has been mentioned that four genera of ticks are found on rats. *Hyalomma aegyptium* is the commonest tick and is particularly abundant during the time when cases of the XK type of typhus are being reported in the Simla Hills. At this time the larvæ and nymphs are found on rodents and possibly on other lower mammals while the adults engorge on goats and sheep. Their presence on wild animals might also explain their relation to the dissemination of this disease. In this respect the other common ticks found on rats, namely *Rhipicephalus sanguineus* and *R. haemaphysaloides*, are also likely vectors of typhus because they have also been recorded on various wild animals. According to Sharif (*loc. cit.*) the latter species has been observed on a leopard at Kasauli, near Kareri lake in the Kangra valley, on sheep and on dog, on man and sheep at Blowali, Malwa Tal, and Muktesar in the hills of the United Provinces. It would, therefore, be interesting to investigate whether experimental transmission of typhus of the XK type is possible with the ticks *Hyalomma* and *Rhipicephalus* and this is in progress.

The other two genera of ticks found on rats, namely *Hæmaphysalis* sp. and *Ixodes* sp., are found in very small numbers. Whether they play any part in the transmission of typhus has yet to be worked out.

The rôle of ticks in the transmission of the X19 form of typhus is very doubtful and they are very much affected by adverse conditions of climate during the cold weather when cases are reported in the Simla Hills.

There is no evidence forthcoming in support of the rat-louse *Polyplax spinulosa* acting as a vector of this disease.

SUMMARY.

1. A total of 2,451 rats (*Muridæ*) and shrews (*Soricidæ*) were trapped at Kasauli and Sabathu in the Simla Hills during the year 1935-36 and their ectoparasites were collected and studied.

2. Amongst the arthropods occurring as external parasites are included the fleas—*Xenopsylla cheopis*, *Ceratophyllus simla*, *Ctenocephalus canis* and *C. felis*, and *Leptopsylla segnis*. The principal mites studied are *Liponyssus bacoti*, *Dermanyssus* sp., *Echinolaelaps echidninus*, and larval forms of the genus *Trombicula*. The ticks are represented by the larvæ and nymphs of *Hyalomma aegyptium*, *Rhipicephalus sanguineus* and *R. hæmaphysaloides*, *Hæmaphysalis* sp., and adults of the genus *Ixodes*. The lice belong to the genus *Polyplax* and the common species met with is *P. spinulosa*.

Details are given of the seasonal distribution of the various ectoparasites on rats and shrews.

3. The possible rôle of fleas, mites, ticks, and lice in the transmission of typhus in the Simla Hills is discussed.

It is emphasized that the larval mite *Trombicula deliensis* and the tick *Hyalomma aegyptium* may possibly be concerned in the transmission of the XK (rural) type of typhus.

Evidence is produced to show that the fleas (*Xenopsylla cheopis* and *Ceratophyllus simla*) are the likely vectors of the X19 (urban) form of typhus in the Simla Hills.

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THE DEFICIENCIES OF THE SOUTH INDIAN DIET.

BY

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IN a previous paper (Aykroyd and Krishnan, 1937*b*) feeding experiments with children and young rats, designed to test the value of various foods in supplementing typical South Indian diets, were reported. Skimmed milk powder was found to be a most effective supplement. Skimmed milk supplies protein of high biological value, the B₂ group of vitamins, and mineral salts including calcium. No definite conclusion about the relative importance of these factors could be drawn from our earlier experiments. It is, however, obviously desirable to define more exactly the deficiencies of Indian diets. In the present experiments, carried out with rats, we have studied the effect of adding various substances, including casein, calcium lactate and yeast, to the 'poor Madrassi diet'.

EXPERIMENTAL.

The basal diet was as follows:—

	Oz.
Raw milled rice	21.00
Dhal arhar (<i>Cajanus indicus</i>)	0.70
Black gram (<i>Phaseolus mungo</i>)	0.70
Brinjal (<i>Solanum melongena</i>)	1.00
Amaranth leaves (<i>Amaranthus gangeticus</i>)	0.50
Raw plantain (<i>Musa paradisiaca</i>)	0.50
Gingelly oil	0.10
Coco-nut	0.05
Meat (mutton)	0.06

The description of this diet as the 'poor Madrassi diet' is justified by the results of detailed diet surveys carried out in various parts of the country. The quantities given correspond roughly to average adult daily intake.

those usually considered sufficient to cover the daily requirements of rats. If the failure of the rats on the basal diet to grow were due primarily to lack of these factors, the addition of the quantities stated should result in a more rapid increase in weight.

DISCUSSION.

In the earlier paper (Aykroyd and Krishnan, 1937*a*) it was pointed out that the young rats used for the growth experiments are well stored with vitamin A, and will grow for 10 weeks on a diet deficient in vitamin A but otherwise complete. None of the supplements given in the present experiments contained vitamin A or carotene in appreciable quantities.

The addition of calcium lactate and dried yeast, unheated or autoclaved, resulted in a marked enhancement of growth. Casein, cystine, flavine, and the vitamin-B₁ preparation had little effect on the weight curves. The present experiments therefore suggest that the effect of the skimmed milk is largely due to its content of calcium and of some factor or factors in the vitamin-B₂ complex.

Two groups of rats on the 'poor Madrassi diet' plus 1.5 oz. (42 g.) of skimmed milk powder showed an average weekly increase in weight of 7.5 g. and 9.4 g., respectively. A similar quantity of casein was given, so that the amount of protein supplied by the latter supplement was in excess of that supplied by the former. Nevertheless growth on the casein-fortified basal diet was very little better than growth on the basal diet alone. The addition of 13.0 mg. of cystine, which would provide about twice as much cystine daily as the basal diet containing casein, had no marked effect on growth.

It is of interest to recall that in Corry-Mann's (1926) well-known series of experiments, which demonstrated the value of adding 'extra' milk to the diet of poorly-fed English children in a charitable institution, the giving of 0.75 oz. (24 g.) of casein daily did not have any 'growth-enhancing' effect. While the value of milk as a supplement to European poor class diets and Indian diets has been abundantly proved, it seems probable that the important operative factor is *not* the additional protein supplied in the form of casein.

As long ago as 1912, McCay showed that the health and physique of Indian peoples whose diet is largely based on rice are inferior to those of peoples consuming wheat and milk. He explained this in terms of the low protein content of rice. It has long been customary, in considering the nutritive value of diets based on rice, to emphasize the importance of the protein factor. Wilson *et al.* (1936), in discussing the results of a diet survey in Calcutta, remark that 'the protein element in nutrition in India may ultimately prove to be the most important and it will certainly be the most difficult to remedy'. This idea may be erroneous.

The inclusion of calcium lactate greatly enhanced growth and improved the general condition of the animals. We regard this as an important observation. It strongly suggests that one of the most serious defects of the South Indian diet is its relative lack of calcium, and that the value of milk is due in large measure to its high calcium content. In all probability controlled experiments on children using calcium lactate instead of milk would show results resembling those obtained in our skimmed milk experiments. The amount of calcium given in these experiments,

based on the usual medicinal dose of calcium lactate, was in excess of that supplied by the 'poor Madrassi diet' supplemented by 1.5 oz. of skimmed milk powder.

The supplement of dried yeast increased growth. Dried yeast contains all the B vitamins, and further analysis was necessary to discover the relative importance of the various factors in the complex. A vitamin-B₁ concentrate, given in adequate daily rat doses, did not improve growth. It is to be observed that true beri-beri is not a common disease in South India. Peripheral neuritis in lactating women, which may be associated with vitamin-B₁ deficiency, is however very common, and it is probable that diets of the 'poor Madrassi' type contain sufficient vitamin B₁ for children and adults in ordinary circumstances, but not enough to make good the drain of lactation.

Pure flavine had very little effect on the growth curve. The addition of black pepper was tried because Narasimhamurthy (1937) found this substance to be very rich in flavine.

Dried yeast, autoclaved for five hours at pH 9.2 and subsequently neutralized, was almost as effective in enhancing growth as unheated dried yeast. Analysis showed the alkaline autoclaved yeast to be devoid of flavine.

We are therefore led to the conclusion that the 'poor Madrassi diet' is deficient in one of the factors in the vitamin-B complex other than vitamin B₁ and flavine. This factor may be vitamin B₆; it is more likely to be the 'alcohol-ether precipitate factor' described by Elvehjem. Koehn and Oleson (1936) and Koehn and Elvehjem (1937). It is possible that this factor is identical with the curative factor in human pellagra, with the factor which prevents black-tongue in dogs, and also with the chick 'anti-pellagra' factor.

A common deficiency disease found in children fed on diets of 'poor Madrassi' type is a stomatitis closely resembling the stomatitis of pellagra. In previous papers this condition has been fully described and its precise relation to diet demonstrated (Aykroyd and Krishnan, 1936, 1937a). It can be cured by dried yeast, unheated or autoclaved. Satisfactory curative experiments with *alkaline* autoclaved yeast have not yet been carried out. The evidence, however, points to the conclusion that a diet largely based on milled rice is deficient in the 'anti-pellagra' factor. Why such a diet tends to produce only one of the symptoms of the pellagra syndrome, and does not lead to the characteristic dermatitis, is a problem of absorbing interest.

The rats on the basal diet plus calcium lactate grew well although not receiving an addition containing the B vitamins. Similarly the diet supplemented by yeast supported fairly good growth although no calcium salt was added. Dried yeast, in the quantity given, would supply negligible amounts of calcium salts. It therefore seems probable that calcium lactate increased the 'availability' or utilization of a factor or factors in the vitamin-B complex already present in small amounts in the basal diet, and vice versa. In this respect growth experiments with a basal diet consisting of natural foods are not precisely parallel to the more familiar type of experiment in which purified synthetic diets are used.

Since dried yeast and calcium, fed separately, enhance the value of the 'poor Madrassi diet' for rats, it might be expected that given in combination they would produce a steeper growth curve. We found, however, that this was not the case.

The average weekly increase in weight when yeast and calcium were given together was no greater than when yeast was given alone, and was actually less than that obtained with calcium alone. Further investigation of this question is necessary.

It must be emphasized that rat-growth experiments of the kind described give only an *indication* of what additions to human diets are likely to prove most valuable. They do, however, give us a line to follow in the difficult task of discovering methods of improving the South Indian diet consistent with economic realities. For example, the experiments with calcium lactate described here have directed our attention to the importance of adding calcium-rich foods to diets largely based on rice. Certain of the millets (e.g., ragi, *Eleusine coracana*) are rich in calcium, and a single meal of millet daily may be of value to children living on a rice staple.

SUMMARY.

1. The value of various supplements to the 'poor Madrassi diet' has been studied by growth tests on rats.
2. Calcium lactate, dried yeast, and dried yeast autoclaved in an alkaline medium effectively supplemented the basal diet.
3. Casein, cystine, flavine, and a vitamin-B₁ concentrate did not enhance the rate of growth.
4. It is probable that the most important defects in diets of the 'poor Madrassi' type are insufficiency of calcium salts and of a heat- and alkali-stable factor in the vitamin-B complex. The identity of this factor is discussed.

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THE NITROGEN COMPLEX OF INDIAN FOODSTUFFS.

CONDIMENTS : PART I. BLACK PEPPER (*PIPER NIGRUM*).

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Piper nigrum is widely grown in India, particularly in the South. The berries are collected while green and dried; when dry they are dark in appearance with a puckered surface. Black pepper is extensively used as a condiment and is held to have carminative properties. In certain parts of the country it is employed in the preparation of 'rasam' and 'sambar' which are courses of the South Indian meal. 'Rasam' is a dilute water-extract of tamarind, salt, and chilly powder to which pepper powder is usually added. 'Sambar' is a similar preparation containing dhal and vegetables; it is much thicker than 'rasam', corresponding in consistency to thick soup.

A considerable portion of the total nitrogen present in certain foodstuffs is in a non-protein form. Pepper is an example of such a foodstuff; it contains non-protein nitrogen to the extent of 82 per cent of the total water-extractible nitrogen. The simple determination of the nitrogen content thus gives a quite erroneous impression of its true protein content. The study of the non-protein nitrogen of foodstuffs of this nature is of practical interest.

It has been found by one of us (Narasimhamurthy, 1937) that pepper is as rich a source of flavine as dried brewer's yeast. The chemistry of the nitrogen complex of pepper has not been studied so far, and since black pepper is widely used in

India it was felt important to carry out the investigation the results of which are described in the present paper.

Previous chemical studies of the nutritive values of proteins have not taken into account the influence of pH on the availability of the nitrogen in foodstuffs. Food when ingested is subjected to various degrees of acidity and alkalinity during its passage through the alimentary tract, and these degrees may be exaggerated in pathological conditions. The influence of pH was also studied in the present investigation.

EXPERIMENTAL.

The dry fruits, obtained locally, were sun-dried and powdered to pass through a 50-mesh sieve: a fairly large quantity of this material was kept ready. Samples were taken as required after thorough mixing. The chemical composition was found to be as follows:—

Moisture, per cent.	Total nitrogen, per cent.	Ether extractives, per cent.	Ash, per cent.	Fibre, per cent.	Carbohydrate (by difference), per cent.
10.78	2.07	6.79	4.40	14.92	50.17

Calcium, phosphorus, and iron were found to be present in the ash in the following proportion:—

	Per cent.
Calcium	10.50
Phosphorus	4.54
Iron	0.38

The following factors are among those influencing nitrogen extraction:—

- (1) the nature of the solvent, (2) the time of extraction, and (3) the hydron concentration of the extracting medium. The bulk of the solvent has also some influence, but this was found to be inconsiderable.

Weighed quantities of the pepper powder were treated with five parts of the several extracting media in Jena-glass bottles at room temperature. In investigating factors (1) and (3), suspensions were rocked in a shaking machine for a period of 15 minutes, and filtered immediately in a Buchner-funnel, the first portion re-filtered to ensure complete removal of all suspended matter in the filtrate. When filtration was very slow, it was conducted in the refrigerator. The filtrates were made up to known volumes and aliquots used to determine the nitrogen extracted in each case. The results are shown in Table I:—

TABLE I.

The influence on nitrogen extraction of (a) saline concentration, (b) the period of extraction, and (c) the pH of the medium.

(a)	{ Saline concentration (per cent)	0	2	4	6	8	
	{ Nitrogen extracted (per cent)	0.242	0.235	0.273	0.235	0.254	
(b)	{ Time (hours) ..	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{4}$	$1\frac{1}{4}$	$1\frac{1}{2}$	
	{ Nitrogen extracted (per cent)	0.266	0.242	0.265	0.240	0.273	
(c)	{ pH	1.0	3.3	8.4	8.0	9.0	3.3 pH and 1.5 per cent saline.
	{ Nitrogen extracted (per cent)	0.270	0.216	0.242	0.215	0.314	0.216

The pH levels that prevail in the alimentary tract alone were considered. In one experiment the influence of both pH and salt concentration was investigated. As already mentioned, pepper is used in the preparation of 'rasam'. The pH and saline concentration of a series of 'rasams' were determined, and it was found that most of the 'rasams' have a pH round about 3.3, and 1.5 per cent salt concentration. It is apparent from Table I that the addition of salt does not exert any appreciable influence on nitrogen extraction; the quantity of the nitrogen extracted at the same pH without salt was very similar.

The next series of experiments was concerned with the determination of the forms in which the nitrogen in pepper exists, i.e., how much is non-protein nitrogen and how much is albumin, globulin, prolamin, and glutelin. Estimations were also made of the amino nitrogen in the non-protein nitrogen fraction. The non-protein nitrogen was estimated directly in the original material by the methods of Mezincescu and Szabo (1936) and Ayres and Lee (1936). Non-protein nitrogen was also estimated in the water-soluble fraction. Albumin, globulin, prolamin, and glutelin were determined by taking 10 g. of the powder and treating it successively for 15 minutes with 50 c.c. of water, 4 per cent saline, 70 per cent alcohol, and 0.1 per cent alkali. The suspensions were shaken well in the rocking machine for 15 minutes, filtered, washed with the respective solvents, and made up to volume. The nitrogen content of each fraction was determined on aliquots. The non-protein nitrogen of each fraction was determined by taking aliquots of each solution and removing the protein by precipitating with enough trichloroacetic acid added to make a 4 per cent solution. The soluble portion was removed by filtration, the residue washed with 2 per cent trichloroacetic acid solution

and the filtrates and washings mixed and made up to volume; nitrogen was estimated on aliquots. Tables II and III give the results:—

TABLE II.

The protein fractions and their non-protein nitrogen (per cent of original material).

Solvent.	Water.	4 per cent saline.	70 per cent alcohol.	0.1 per cent NaOH.	Residue (by difference).
Nitrogen	0.266	0.0426	0.149	0.08	1.48
Non-protein nitrogen	0.253	Nil	Nil	Nil	..

TABLE III.

The non-protein nitrogen and amino nitrogen of pepper (expressed as per cent of original material).

	Precipitation with 4 per cent trichlor-acetic acid.	Precipitation with sodium tungstate (method of Ayres and Lee).	Precipitation with trichlor-acetic acid (method of Mezincescu and Szabo).	Van Slyke's gaso-metric method.
Non-protein nitrogen ..	0.253	0.207	0.255	..
Amino nitrogen	0.149	..	0.128

Table II shows that the amount of albumin and globulin nitrogen present was found to be very small. In practical nutrition we are concerned with the entire seed. Hence it was considered better to study nitrogen distribution in the whole seed. Accordingly about 50 g. of pepper powder were de-fatted in the Soxhlet apparatus and dried. Samples of this fat-free dry powder were used for the study of the hydrolysis products and for the estimation of tyrosine and tryptophane. The portion used for studying the nitrogen distribution was hydrolysed with 25 per cent hydrochloric acid at 110°C. under reflux for 48 hours. Van

Slyke's (1911) method of analysis modified by Plimmer and Rosedale (1925) was then followed. Cystine was estimated by the method of Plimmer and Lowndes (1927) on the basic fraction of the hydrolysate. Tyrosine and tryptophane were estimated on a separate sample by the colorimetric method of Folin and Marenzi (1929). The results are tabulated in Table IV :—

TABLE IV.

Nitrogen distribution of black pepper.

(Expressed as per cent of the nitrogen of the hydrolysate from fat-free material.)

Acid soluble humin nitrogen.	Amide nitrogen.	BASIC FRACTION.		NON-BASIC FRACTION.		TOTAL.
		Amino nitrogen.	Non-amino nitrogen.	Amino nitrogen.	Non-amino nitrogen.	
3.35	17.00	8.68	3.385	58.08	9.58	100.075
Nitrogen as :— Arginine .. 1.118 Histidine .. 3.800 Cystine .. 1.280 Lysine .. 5.867						

Tyrosine and tryptophane content of black pepper.

(Expressed as per cent of the original material.)

Tyrosine.	Tryptophane.
0.929	0.70

DISCUSSION.

Nitrogen compounds, when ingested, are broken down under the influence of the intestinal secretions into amino acids, in which form the body utilizes them.

The degradation of protein, like several other chemical processes occurring in solution, is more rapid the more soluble the nitrogenous compounds. In pepper, though only 12 per cent of the total nitrogen is water soluble, most of it is in non-protein form; more than half of this fraction is made up of simple amino acids, which can be readily utilized in the system. It is to be expected on this score that pepper may have a high biological value. This question must, however, be studied by biological experiments.

A point of interest is the amino-acid make-up of the nitrogen complex of pepper. The amino acids which are most essential for growth and maintenance are all present in this foodstuff (see Table IV). Lysine, histidine, and cystine are present in appreciable quantities. It is of interest to compare the composition of pepper in this respect with that of the proteins of the other foods with which pepper is generally consumed, e.g., the staple cereal grains of India, such as wheat, rice, cholam, and ragi. The nitrogen distribution in these grains are shown in Table V:—

TABLE V.

The amino-acid composition of black pepper and that of protein obtained from various cereals compared.*

	Eleusin from <i>Eleusine coracana</i> (ragi).	Kaffirin from <i>Andropogan sorghum</i> (cholam).	Leucosin from <i>Triticum vulgare</i> (wheat).	Oryzenin from <i>Oryza saliva</i> (rice).	Nitrogen complex of <i>Piper nigrum</i> (pepper).
Arginine ..	2.60	3.92	5.94	9.15	1.18
Histidine ..	2.69	1.71	2.83	3.32	4.40
Cystine ..	?	1.23	..	1.26	1.48
Lysine ..	0.64	2.48	2.75	4.26	6.81
Total protein per cent of the seed.	7.1	10.42	11.77	6.44	12.9

* These data regarding cereals were taken from Winton and Winton (1932). Csonka (1937) in a recent publication gives lower values for the amino acids in wheat.

Black pepper contains more histidine, cystine, and lysine than the proteins of the common cereals listed above. It may, therefore, have a useful 'supplementary' value when consumed in combination with cereal foods, notably ragi, which is very deficient in cystine and lysine.

SUMMARY.

1. An investigation of the chemical composition and the nitrogen distribution in the nitrogen complex of Indian black pepper (*Piper nigrum*) has been carried out.
2. Non-protein nitrogen constitutes 82 per cent of the water and saline-soluble nitrogen fractions of black pepper ; of this over 56 per cent is in the amino condition.
3. Black pepper contains appreciable amounts of all the important amino acids ; its richness in lysine and histidine suggests that it may be of more value in human nutrition than has hitherto been realized.

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THE RELATIVE VALUE OF THE PROTEINS OF CERTAIN FOODSTUFFS IN NUTRITION.

Part III.

THE BIOLOGICAL VALUE OF THE PROTEINS OF VARIOUS PULSES, OIL-SEEDS, NUTS, AND SKIMMED MILK, STUDIED BY THE BALANCE-SHEET METHOD.

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AN earlier paper in this series (Part I) (Swaminathan, 1937) dealt with the value of the proteins of 13 common foodstuffs (5 cereals, 3 pulses, and 5 vegetables) investigated by the balance-sheet method. This paper reports further similar studies of the proteins of 14 foodstuffs, including pulses, oil-seeds, and nuts.

THE COMPOSITION OF FOODSTUFFS AND EXPERIMENTAL DIETS.

The pulses, after air-drying, were powdered to pass through a fine-mesh sieve. Three of those tested—dhal arhar (red gram), black gram, and Bengal gram—could easily be freed from their husks and were powdered in the husked state; the remaining 3 were powdered with their husks. The oil-seeds and nuts were dried in an air oven at a temperature not exceeding 70°C., and de-fatted by ether. The residue, almost fat-free, was first dried in the sun and subsequently in an air oven below 70°C.; finally it was reduced to fine powder. De-fatting was carried in the case of nuts and oil-seeds, since the presence of large quantities of fat makes satisfactory powdering difficult. The vegetables tested were dried under carefully regulated conditions as described in the previous paper (Swaminathan, *loc. cit.*).

The level of protein intake was kept at approximately 10 per cent in the diets containing the pulses and oil-seeds, and at 5 per cent in those containing the cereals

382 *The Relative Value of Proteins of Certain Foodstuffs in Nutrition.*

and pulses. The moisture and crude protein content of the powdered foodstuffs are given in Table I and the composition of the test diets in Table II:—

TABLE I.

Moisture and 'crude protein' content of test materials.

Name of foodstuff.	Botanical name.	Moisture, per cent.	'Crude protein', per cent.
Italian millet	<i>Setaria italica</i>	11.23	10.04
Bengal gram	<i>Cicer arietinum</i>	11.20	22.10
Black gram	<i>Phaseolus mungo</i>	10.87	23.50
Cow gram	<i>Vigna catieng</i>	12.00	24.12
Field beans	<i>Dolichos lablab</i>	9.60	24.63
Horse gram	<i>Dolichos biflorus</i>	11.81	22.12
Dhal arhar (red gram) ..	<i>Cajanus indicus</i>	12.52	22.65
Coco-nut	<i>Cocos nucifera</i>	36.28	4.47
„ de-fatted	„ „	4.16	16.20
shew-nut	<i>Anacardium occidentale</i>	5.89	21.19
„ de-fatted	„ „	3.45	31.77
Gingelly seeds	<i>Sesamum indicum</i>	5.08	18.33
„ de-fatted	„ „	3.12	28.98
Ground-nut	<i>Arachis hypogea</i>	7.92	26.72
„ de-fatted	„ „	2.84	45.73
Cluster beans	<i>Cyamopsis psoralioides</i>	82.45	3.67
„ dry powder	„ „	8.30	19.46
Sesbania leaves	<i>Sesbania grandiflora</i>	76.83	8.40
„ dry powder	„ „	8.24	31.85
Skimmed milk powder ..	—	4.10	38.04

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Table III.

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experiments and the calculated biological

TABLE II.

Percentage composition of the diets.

	Italian millet.	Bengal gram.	Black gram.	Cow gram.	Field beans.	Horse gram.	Dhal arhar (red gram).	Coco-nut.	Cashew-nut.	Gingelly seeds.	Ground-nut.	Cluster beans.	Sesbania leaves.	Skimmed milk powder.
Foodstuff tested ..	50.0	45.0	43.0	42.0	41.0	45.0	44.0	62.0	31.4	31.1	22.0	51.0	31.0	26.4
Starch ..	34.4	38.6	40.4	41.2	42.1	38.6	39.5	18.7	48.1	44.5	56.0	31.3	49.7	52.8
Beef dripping ..	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Salt mixture ..	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cod-liver oil ..	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Calcium carbonate ..	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Percentage of N (dry weight).	0.8512	1.6512	1.6752	1.6368	1.6336	1.6432	1.6336	1.6512	1.6512	1.6448	1.6544	0.8368	0.8592	0.8528
* Crude protein ' per cent N \times 6.25 (dry weight)	5.32	10.32	10.47	10.23	10.21	10.27	10.21	10.32	10.32	10.28	10.34	5.23	5.37	5.33

(With 4 c.c. of an aqueous solution of yeast extract corresponding to 1 g. of dried yeast daily to each rat.)

TABLE III.

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
Period 1.— <i>Nitrogen-free diet</i> ($N = 0.0092$ per cent).										
1	224	213	14.80	..	43.2	21.8
2	244	231	15.50	..	46.4	23.6
3	191	186	12.85	..	37.5	20.5
4	193	187	12.55	..	40.2	17.2
5	197	191	14.20	..	42.7	17.6
Period 2.— <i>Skimmed-milk-powder diet</i> ($N = 1.6592$ per cent).										
1	222	235	16.55	274.6	94.5	58.9	200.8	251.6	80	92
2	239	255	19.83	329.0	92.8	60.2	250.2	294.7	85	90
3	190	200	19.25	319.4	74.2	60.3	246.3	280.9	88	88
4	197	210	16.05	266.3	87.2	53.4	184.3	232.5	79	87
5	190	195	17.48	290.0	88.2	42.5	225.2	267.5	84	92
Average ..									83	90

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 3.—Skimmed-milk-powder diet (N = 0.8528).</i>										
SERIES 1:										
1	230	244	20.15	171.2	59.2	38.0	140.9	156.4	90	91
2	258	272	21.45	183.0	59.9	38.4	158.9	170.5	93	93
3	200	210	18.40	157.0	53.2	40.6	124.6	138.2	90	88
4	198	210	15.32	130.7	55.7	27.3	106.3	123.0	86	94
5	197	204	16.42	140.1	60.9	39.7	105.4	120.4	88	86
							Average ..		89	90
<i>Period 4.—Sesbania-leaves diet (N = 0.8592 per cent).</i>										
SERIES 1:										
1	228	212	10.58	91.0	73.5	33.5	50.9	80.7	63	89
2	256	240	8.98	73.2	71.4	32.2	43.8	66.9	66	91
3	197	185	8.68	74.7	62.0	30.3	43.8	66.2	66	89
4	199	187	10.03	86.3	64.8	38.1	42.0	67.8	62	79
5	198	193	13.40	116.2	80.2	45.5	56.4	90.7	62	78
							Average ..		64	85

TABLE III—*contd.**(Figures of intake and excretion represent daily averages.)*

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 5.—Nitrogen-free diet (N = 0.0092 per cent).</i>										
SERIES 1:										
1	222	220	18.58	..	44.1	24.6
2	247	244	19.78	..	50.1	28.1
3	185	182	15.70	..	41.7	23.1
4	197	186	12.85	..	37.8	22.0
5	190	185	13.65	..	49.0	22.3
<i>Period 1.—Nitrogen-free diet (N = 0.0092 per cent).</i>										
SERIES 2:										
15	130	118	8.55	..	32.9	11.8
16	128	122	8.10	..	34.7	12.2
17	144	135	9.48	..	39.2	16.4
18	142	133	8.70	..	46.6	16.3
19	162	150	10.63	..	52.5	20.7

TABLE III—*could*,

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 2.—Dhal-arhar (red-gran) diet (N = 1.6336 per cent).</i>										
SERIES 2 :										
15	116	115	10.35	169.1	81.9	66.2	73.9	117.4	63	69
16	120	123	9.88	161.4	76.6	47.6	91.0	128.3	71	70
17	132	132	8.70	142.1	76.3	58.6	66.1	101.6	65	72
18	127	128	10.35	169.1	78.0	62.6	101.1	125.3	81	74
19	154	152	9.90	161.8	84.3	59.4	101.5	126.1	80	78
								Average ..	72	75
<i>Period 3.—Bengal-gran diet (N = 1.6512 per cent).</i>										
SERIES 2 :										
15	128	139	11.98	197.8	111.0	37.6	102.1	174.7	58	83
16	133	139	11.05	182.4	93.5	43.3	99.4	153.6	65	84
17	143	143	8.90	146.9	97.0	33.7	75.1	131.3	57	90
18	148	152	13.73	226.7	133.7	49.9	115.7	195.6	59	86
19	183	187	16.03	264.7	127.8	55.1	165.2	233.3	71	88
								Average ..	62	86

TABLE III—*contd.*
(*Figures of intake and excretion represent daily averages.*)

Rat series and number.	Initial body- weight, g.	Final body- weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
Period 4.— <i>Black-gram diet (N = 1.6752 per cent).</i>										
SERIES 2:										
15	130	136	13.08	219.1	103.6	59.2	109.2	174.4	63	80
16	125	130	12.80	214.4	112.0	59.1	97.1	169.8	57	79
17	135	133	12.45	208.5	111.0	63.8	92.6	162.8	57	78
18	150	157	14.03	235.0	113.8	74.6	119.2	179.2	67	76
19	175	177	16.03	268.5	127.4	81.3	143.2	210.9	68	79
Average ..									62	78

Period 5.— <i>Nitrogen-free diet (N = 0.0092 per cent).</i>										
SERIES 2:										
15	138	135	12.18	..	43.8	17.2
16	132	134	12.80	..	43.8	16.7
17	150	147	13.10	..	42.4	19.8
18	168	158	13.73	..	60.9	21.3
19	205	195	18.53	..	66.9	26.3

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 6.—Cluster-beans diet (N = 0.8368 per cent).</i>										
SERIES 2 :										
15	132	124	14.73	123.3	88.6	58.4	39.5	82.0	48	67
16	130	118	13.50	113.0	86.8	45.7	45.6	84.9	54	75
17	142	132	11.64	97.4	80.9	43.0	37.9	73.6	51	76
18	150	135	12.55	105.1	102.2	52.3	33.9	74.6	45	71
19	175	177	15.53	130.0	109.2	55.2	57.9	101.0	57	78
								Average ..	51	73
<i>Period 7.—Field-beans diet (N = 1.6336 per cent).</i>										
SERIES 2 :										
15	130	110	9.43	154.1	112.7	60.5	44.1	110.7	40	72
16	130	110	10.15	165.9	119.4	65.4	46.2	118.1	39	71
17	142	132	9.53	155.7	106.8	56.7	56.6	118.2	48	76
18	150	140	10.50	171.6	148.8	53.5	52.6	139.9	38	82
								141.3	40	81
								Average ..	41	76

TABLE III—*contd.**(Figures of intake and excretion represent daily averages.)*

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 8.—Ground-nut diet (N = 1.6544 per cent).</i>										
15	117	125	12.13	200.6	131.6	36.9	95.3	180.8	53	90
16	132	142	12.60	208.4	136.9	41.9	93.3	184.1	51	88
17	145	157	13.70	226.7	123.2	37.3	130.6	208.6	63	92
18	147	167	14.63	242.0	164.1	46.9	114.3	216.9	53	90
19	192	209	19.48	322.2	176.1	52.4	186.0	296.0	63	92
Average ..									57	90

<i>Period 9.—Nitrogen-free diet (N = 0.0092 per cent).</i>										
15	125	116	11.38	..	48.3	16.9
16	145	139	12.78	..	51.1	18.4
17	158	152	13.95	..	48.0	18.6
18	168	160	13.73	..	62.0	22.3
19	211	204	11.93	..	65.5	26.0

TABLE III—*contd.**(Figures of intake and excretion represent daily averages.)*

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Facal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 12.—Cow-grass diet (N = 1.6368 per cent).</i>										
SERIES 2:										
15	182	182	13.78	225.6	138.3	59.0	99.8	184.0	54	82
16	200	200	16.53	270.6	167.0	89.5	87.0	200.6	43	76
17	200	200	14.68	240.3	140.0	66.3	89.9	193.1	47	80
18	212	211	13.95	228.3	156.8	89.3	69.0	162.0	43	71
19	245	246	16.50	270.1	193.5	83.5	86.0	212.7	40	79
Average ..									45	78

SERIES 2: *Period 13.—Nitrogen-free diet (N = 0.0092 per cent).*

15	195	183	11.58	..	59.9	17.8
16	212	205	16.45	..	55.6	20.5
17	210	195	13.63	..	52.1	19.5
18	240	230	15.48	..	65.5	23.7
19	220	204	12.23	..	68.1	26.2

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 14.—Horse-grass diet (N = 1.6432).</i>										
SERIES 2 :										
15	180	175	12.80	210.3	100.8	65.9	122.0	162.7	75	77
16	205	208	13.48	221.5	117.6	94.1	86.9	148.3	59	67
17	207	200	12.35	202.9	104.3	88.1	84.0	134.9	62	66
18	220	215	12.45	204.5	119.7	55.1	118.0	173.4	68	84
19	204	200	12.60	207.0	120.4	81.3	97.7	151.1	65	73
								Average ..	66	73
<i>Period 15.—Italian-wheat diet (N = 0.8512).</i>										
SERIES 2 :										
15	175	180	13.63	116.0	72.8	30.4	91.2	103.9	88	90
16	210	215	15.83	134.7	93.8	31.0	87.0	124.6	70	93
17	210	217	15.55	132.3	86.8	36.4	82.6	116.0	71	88
18	220	232	18.68	159.0	102.9	41.3	103.1	141.7	73	89
19	200	205	14.98	127.5	90.3	33.1	96.5	119.8	81	94
								Average ..	77	91

TABLE III—*concd.*
(*Figures of intake and excretion represent daily averages.*)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
Period 16.—Coco-nut diet (N = 1.6512 per cent).										
SERIES 2 :										
15	177	182	14.04	231.7	102.2	38.4	169.5	211.6	80	91
16	212	215	13.94	230.1	110.6	41.9	154.7	209.1	74	91
17	207	210	14.04	231.7	111.5	35.3	158.4	216.5	73	93
18	227	231	13.27	220.2	106.8	31.9	169.8	212.3	80	97
19	204	208	14.04	231.7	113.6	33.2	177.3	223.9	79	97
Average ..									77	94
Period 17.—Nitrogen-free diet (N = 0.0092).										
SERIES 2 :										
15	178	171	14.95	..	60.2	18.8
16	210	204	13.93	..	56.7	21.3
17	205	202	13.60	..	54.3	20.6
18	225	218	15.95	..	63.0	24.2
19	200	191	14.30	..	65.8	24.6

DISCUSSION.

Cereals.—Comparing values obtained for cereals in this and previous studies, Italian millet has a biological value (77) higher than that of wheat (66), but somewhat lower than those of rice, cambu (*Pennisetum typhoideum*), cholam (*Sorghum vulgare*), and ragi (*Eleusine coracana*), which were found to be 80, 83, 83, and 89, respectively.

Pulses.—Of the pulses studied in this investigation, dhal arhar (red gram) stands highest in biological value (72). Niyogi *et al.* (1931a) reported a value of 74 for the proteins of this pulse. Miller and Robbins (1936) have found by growth experiments that dhal arhar (red gram) is deficient in cystine and tryptophane.

The value obtained in the case of horse gram (66) corresponds to that recorded by Niyogi *et al.* (1931b). In the present study the proteins of black gram and Bengal gram were found to be of equal value (62); Niyogi *et al.* (1931b, 1932b) reported 78 and 64, respectively. The proteins of cow gram and field beans were found to have a low biological value of 45 and 41, respectively. These figures are lower than those of Niyogi *et al.* (1931b, 1932a) which were 72 and 57.

The biological values of the pulse proteins so far studied by the author range themselves in the following descending order : red gram, horse gram, black gram, Bengal gram, soya bean, green gram, cow gram, field beans, and lentil.

Nuts and oil-seeds.—The proteins of nuts and oil-seeds appear to be superior to those of pulses and perhaps comparable to those of meat and fish. Eddy and Eckman (1923), comparing pea-nut protein with meat protein, concluded that 'when the protein supplementing power of pea-nut flour is compared with that of muscle proteins by feeding rations so constituted as to contain only about 10 per cent of protein, 6 to 7 per cent of this protein being contributed by wheat flour and the rest by pea-nut flour or meat residues, respectively, and when these rations are further supplemented with 3 per cent of butter-fat, 4 per cent salts, and brought to nearly equal calorie values per gramme, the pea-nut flour proves slightly superior to meat as a growth producer and markedly superior for promoting reproduction'.

Of the four nuts and oil-seeds investigated, the proteins of coco-nut have the highest biological value (77). Mitchell and Villegas (1923), on the other hand, obtained a lower biological value of 58. In the work of Johns, Finks and Gersdorff (1919) coco-nut protein was found by feeding experiments to furnish all the essential amino acids. Sherman (1933) observes, regarding the proteins of coco-nut, that 'since the chemical composition shows it to be fairly high in lysine, it should be of value as a supplement to the grain proteins. Its lysine content of about 6 per cent gives it rank in this respect somewhat below the milk proteins and about equal to the proteins of egg, meat, fish, and gelatin'.

The proteins of cashew-nut are also of high biological value (72). Chatterji (1930) found that cashew-nut proteins produced good growth in young rats, while Damodaran and Sivaswamy (1936) showed by chemical analysis that the chief protein of cashew-nut is a biologically complete protein. The proteins of gingelly seeds have not been investigated before. A fairly high biological value of 67 was obtained. The proteins of ground-nut were found to have a lower biological value (57) than the other three nuts. Pian (1930) and Mitchell *et al.* (1936), using young rats, reported biological values of 58 and 59, respectively; but Eddy and Eckman

(*loc. cit.*) found that the proteins of a mixture of wheat and ground-nut supported slightly better growth than that produced by a mixture of wheat and mutton proteins. Shiba and Koyama (1923) found pea-nut protein somewhat more efficient for growth than soya-bean protein.

Vegetables.—In the previous communication (Swaminathan, *loc. cit.*) the biological values of the proteins of five vegetables were reported. It was found that in general vegetables contained proteins of fairly high biological value. In the present investigation it was found that sesbania leaves contain proteins of good quality, with a biological value of 64. The proteins of cluster beans were found to have a lower biological value (51); in this respect cluster beans resemble other beans such as field beans, navy beans, etc.

Skimmed milk powder.—The proteins of skimmed milk powder have been the subject of numerous investigations in the past. Mitchell *et al.* (1924, 1926, 1935) have studied this problem by the balance-sheet method using young rats. They obtained values varying from 82 to 85 when this level of protein intake was 10 per cent. In the present investigation adult rats were used, and biological values of 89 and 83 were obtained at 5 and 10 per cent levels of protein intake, respectively.

TABLE IV.

The available or net protein content of the foodstuffs investigated.

Name of foodstuff.	Moisture, per cent.	'Crude protein', per cent.	Level of protein intake, per cent.	Biological value, per cent.	Digestibi- lity co- efficient.	Available or net protein content, per cent.
Italian millet	11.23	10.02	5	77	91	7.02
Bengal gram	11.20	22.10	10	62	86	11.78
Black gram	10.87	23.50	10	62	78	11.36
Cow gram	12.00	24.12	10	45	78	8.47
Field beans	9.60	24.63	10	41	76	7.67
Horse gram	11.81	22.12	10	66	73	10.65
Dhal arhar (red gram) ..	12.52	22.65	10	72	75	12.23
Coco-nut de-fatted powder ..	4.16	16.20	10	77	94	11.73
„ fresh	36.28	4.47	10	77	94	3.24
Cashew-nut de-fatted powder	3.45	31.77	10	72	90	20.59
„ fresh	5.89	21.19	10	72	90	13.73

The figures given for materials described as fresh are calculated from the data obtained with the de-fatted materials.

TABLE IV—*conold.*

Name of foodstuff.	Moisture, per cent.	'Crude protein', per cent.	Level of protein intake, per cent.	Biological value, per cent.	Digestibi- lity co- efficient.	Available or net protein content, per cent.
Gingelly seeds de-fatted powder	3.12	28.98	10	67	85	16.50
„ fresh	5.08	18.33	10	67	85	10.44
Ground-nut de-fatted powder	2.84	45.73	10	57	90	23.46
„ fresh	7.92	26.72	10	57	90	13.71
Cluster beans dry powder ..	8.30	19.46	5	51	73	7.24
„ „ fresh	82.45	3.67	5	51	73	1.37
Sesbania leaves dry powder ..	8.24	31.85	5	64	85	17.33
„ fresh	76.83	8.40	5	64	85	4.55
Skimmed milk powder ..	4.10	38.04	10	83	90	28.42
„ „ „ ..	4.10	38.04	5	89	90	30.47

The figures given for materials described as fresh are calculated from the data obtained with the de-fatted materials.

The net or available protein content of the foodstuffs.—In Part I of this series the 'available or net protein content' of 13 foodstuffs investigated was given, and the importance of this co-efficient was discussed. The available or net protein content of the foodstuffs studied in this investigation is given in Table IV. It may be pointed out that the figure for 'net protein content', calculated according to the usual method, may vary with the level of protein intake. For example, in the case of skimmed milk powder, which contains 38.04 per cent of 'crude protein', 30.47 per cent is 'available' when the level of protein intake is 5 per cent and only 28.42 per cent when the level of protein intake is 10 per cent. With the lower level of protein intake the biological value and hence the 'available or net protein' is higher.

SUMMARY.

1. The biological value and the digestibility co-efficients of 14 common foodstuffs have been determined by the nitrogen-balance method using adult rats.

2. The proteins of Italian millet were found to be superior to those of wheat and slightly inferior to those of rice, ragi, cambu, and cholam, the biological value and digestibility co-efficient obtained being 77 and 91, respectively.

3. At a 10 per cent level of protein intake, the digestibility co-efficients and biological values of the proteins of the six pulses investigated were as follows: dhal arhar (red gram), 75 and 72; horse gram, 73 and 66; black gram, 78 and 62; Bengal gram, 86 and 62; cow gram, 78 and 45; and field beans, 76 and 41.

4. At a 10 per cent level of protein intake the same values for the proteins of four nuts and oil-seeds were: coco-nut, 94 and 77; cashew-nut, 90 and 72; gingelly seeds, 85 and 67; and ground-nut, 90 and 57.

5. At a 5 per cent level of protein intake, the values of the proteins of two vegetables were: sesbania leaves, 85 and 64; and cluster beans, 73 and 51.

6. The digestibility co-efficients and the biological values of the proteins of skimmed milk powder at two levels of protein intake were as follows: at a 5 per cent level of protein intake, 90 and 89; at a 10 per cent level of protein intake, 90 and 83.

7. The 'available or net protein content' of the 14 foodstuffs investigated were also calculated.

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THE RELATIVE VALUE OF THE PROTEINS OF CERTAIN FOODSTUFFS IN NUTRITION.

Part IV.

SUPPLEMENTARY VALUES: STUDIED BY (A) THE BALANCE-SHEET METHOD, USING ADULT RATS, AND (B) THE GROWTH METHOD, USING YOUNG RATS.

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SINCE the biological value of protein largely depends on its amino-acid composition, the proteins of two or more foodstuffs may be able to supply one another's deficiencies, so that a mixture containing equal quantities of two proteins may have a higher value than the arithmetical mean of their individual biological values. The total protein in an ordinary diet consists of a mixture of proteins derived from foodstuffs of both vegetable and animal origin. Previous papers have described investigations on the biological value of the proteins of common Indian foods (Swaminathan, 1937*a, b, c*). It was felt important to follow up this work by the study of the effect of various proteins in supplementing each other. In this investigation particular attention has been given to supplementary relations between the proteins of rice and those of other common foods.

(A) EXPERIMENTS BY THE BALANCE-SHEET METHOD.

Only a few investigations of the supplementary relations between various proteins have been made by the nitrogen balance-sheet method. McCay (1912) worked out the 'co-efficient of absorption' of the mixed proteins in various Indian diets by subtracting the nitrogen lost in the faeces from the nitrogen intake. In his experiments, no account was taken of the loss of food nitrogen in urine, which is generally greater than the faecal loss and hence more important. Mitchell (1924) and Mitchell and Carman (1926) demonstrated that the proteins of milk supplement

those of maize, and that a supplementary relation exists between the proteins of white flour and those of milk, eggs, veal, and beef. Adolph and Cheng (1935) studied the supplementary relationship between the proteins of certain cereals and pulses, viz., wheat, maize, millet, soya bean, cow pea, and kaoliang by the nitrogen balance-sheet method, and found that the proteins of a mixture of maize, millet, and soya bean had, among other mixtures tested, the highest biological value (83). Lan (1936), on the other hand, could not confirm the findings of Adolph and Cheng (*loc. cit.*), obtaining a lower value (73).

Experimental.

The composition of the foodstuffs and diets used are shown in Tables I and II. With the exception of soya bean, the pulses used were husked. The level of protein intake was kept at approximately 8 per cent, 4 per cent being derived from rice and 4 per cent from the various pulses and skimmed milk powder. In one experiment a mixture of vegetable foodstuffs was investigated (diet No. 1). The methods employed were the same as those described in a previous communication (Swaminathan, 1937*a*). The data regarding the metabolism experiments and the calculation of the biological values are given in Table III.

TABLE I.

Moisture and 'crude protein' content of the foodstuffs used.

Name of foodstuff.	Botanical names.	Moisture present.	'Crude protein' (N \times 6.25), per cent.
Raw milled rice	<i>Oryza sativa</i>	12.96	6.65
Red gram (<i>Dhal arhar</i>) ..	<i>Cajanus indicus</i>	12.84	22.24
Soya bean	<i>Glycine hispida</i>	8.08	40.40
Bengal gram	<i>Cicer arietinum</i>	12.20	22.48
Black gram	<i>Phaseolus mungo</i>	10.87	24.12
Green gram	<i>Phaseolus radiatus</i>	10.42	23.80
Skimmed milk powder ..	—————	4.10	38.04

TABLE II.

Percentage composition of the diets.

	Rice and red-gram diet.	Rice and skimmed-milk-powder diet.	Rice and soyabean diet.	Rice and Bengal-gram diet.	Rice and black-gram diet.	Rice and green-gram diet.	Rice, red-gram, and skimmed-milk-powder diet.	Rice, soyabean, and skimmed-milk-powder diet.	Rice, black-gram, and skimmed-milk-powder diet.	Rice, Bengal-gram, and skimmed-milk-powder diet.	Rice, green-gram, and skimmed-milk-powder diet.
Raw milled rice ..	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Pulse ..	18.0	..	10.0	18.0	17.0	17.0	5.0	5.0	8.5	9.0	8.5
Skimmed milk powder	..	10.4	5.2	5.2	5.2	5.2	5.2
Starch ..	10.0	16.1	16.5	10.0	11.0	11.0	5.0	16.1	13.5	13.0	13.5
Salt mixture ..	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Calcium carbonate ..	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Beef dripping ..	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Cod-liver oil ..	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Percentage of N (dry weight).	1.323	1.346	1.371	1.310	1.323	1.314	1.266	1.310	1.290	1.328	1.326
* Crude protein, per cent (N X 6.25) dry weight.	8.27	8.41	8.57	8.10	8.27	8.21	8.10	8.19	8.06	8.30	8.29

(With 4 c.c. of an aqueous solution of yeast extract corresponding to 1 g. of dried yeast daily to each rat.)

TABLE II—*concl'd.**Percentage composition of diet No. 1.*

Raw milled rice	60.0
Red gram	10.0
Black gram	10.0
Powdered amaranth leaves ..	2.0
Powdered brinjal (<i>Solanum melogena</i>).	4.0
Coco-nut powder	3.2
Salt mixture	4.0
Calcium carbonate	0.8
Gingelly oil	13.0
Cod-liver oil	3.0
Percentage of N (dry weight)	1.686
'Crude protein' (N \times 6.25) dry weight.	10.54

Note.—The amaranth leaves powder, brinjal powder, and the coco-nut powder used were the same as those used in the previous experiments.

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
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SERIES I : *Period 5.—Nitrogen-free diet (N = 0.0092 per cent).*

6	223	215	20.38	..	68.3	23.8
7	205	200	20.58	..	64.4	27.2
8	181	172	15.70	..	46.6	16.6
9	173	167	16.18	..	46.9	21.0

SERIES I : *Period 6.—Rice and Bengal-grain diet (N = 1.310 per cent).*

6	210	217	21.13	276.9	133.7	61.4	170.0	238.4	71	86
7	195	201	18.08	236.9	130.5	52.9	135.8	206.8	66	87
8	160	171	14.93	195.6	99.5	51.4	105.3	160.5	66	82
9	155	163	16.23	212.6	112.7	52.0	111.8	179.7	62	85
Average ..									66	85

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
<i>Period 9.—Nitrogen-free diet (N = 0.0092 per cent).</i>										
SERIES 1 :										
6	235	230	21.18	..	62.3	21.9
7	215	210	17.90	..	54.3	18.4
8	162	160	16.25	..	42.4	16.0
9	167	162	17.00	..	42.7	17.2
<i>Period 1.—Nitrogen-free diet (N = 0.0092 per cent).</i>										
SERIES 2 :										
10	139	135	12.75	..	54.5	15.3
11	159	154	13.48	..	62.0	18.9
12	150	145	12.35	..	50.8	17.9
13	155	147	10.98	..	51.1	18.2
14	145	139	12.78	..	50.1	16.2

TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat series and number.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, mg.	Urinary N, mg.	Faecal N, mg.	Body N saved, mg.	Food N absorbed, mg.	Biological value, per cent.	Digestibility co-efficient, per cent.
SERIES 2: <i>Period 4.—Rice, black-gram, and skimmed-milk-powder diet (N = 1.290 per cent).</i>										
10	200	213	15.15	195.4	87.5	37.6	145.0	173.9	83	89
11	200	210	12.78	164.9	90.3	39.5	119.9	144.7	83	88
12	200	208	13.30	171.6	95.9	26.5	119.7	163.2	73	95
13	205	210	13.28	171.3	79.8	25.8	136.5	163.9	83	96
14	202	210	13.88	179.1	88.9	28.5	130.8	167.8	78	94
Average ..									80	92

SERIES 2: *Period 5.—Nitrogen-free diet (N = 0.0092 per cent).*

10	220	205	15.32	..	62.7	16.8
11	220	200	13.90	..	69.0	19.7
12	208	198	12.18	..	53.9	18.2
13	220	205	14.78	..	53.6	18.6
14	219	205	15.33	..	53.6	18.1

Discussion.

In previous investigations (Swaminathan, 1937*a, b, c*) the biological values of the proteins of various cereals, pulses, nuts, oil-seeds, and of skimmed milk powder were determined. The level of protein intake was kept at approximately 5 per cent in the diets containing the cereals and vegetables, and at 10 per cent in those containing the pulses, nuts, oil-seeds, and skimmed milk powder. In the present study the level of protein intake was kept at approximately 8 per cent, 4 per cent of which was derived from the cereals and 4 per cent from the pulses or skimmed milk powder. The experiments fall into two groups: in the first group proteins contained in two foodstuffs (4 per cent from the cereals and 4 per cent from the pulses or skimmed milk powder) were tested; in the second group protein contained in three foodstuffs (4 per cent from the cereals, 2 per cent from the pulses, and 2 per cent from skimmed milk powder).

GROUP I. *Proteins from mixtures of two foodstuffs.*—Of the six mixtures investigated (rice and skimmed milk powder, rice and red gram, rice and soya bean, rice and black gram, rice and Bengal gram, and rice and green gram) the proteins contained in the rice and skimmed-milk-powder mixture were found to possess the highest biological value (80). Next in order of merit came the proteins of the mixture of rice and red gram, the biological value obtained being 76. In the case of other four mixtures biological values were as follows: rice and soya bean, 68; rice and Bengal gram, 66; rice and black gram, 61; and rice and green gram, 56.

GROUP II. *Proteins from mixtures of three foodstuffs.*—In the five diets containing proteins from mixtures of rice and different pulses (whose biological values were determined in the first group of experiments), 2 per cent of the pulse proteins were replaced by an equal percentage of protein from skimmed milk powder. It was found that the proteins contained in the mixture of rice, red gram, and skimmed milk powder had the highest biological value (90). Next in order of merit came the proteins of the mixture of rice, soya bean, and skimmed milk powder, the biological value obtained being 86. The biological values of the proteins of the other mixtures tested were as follows: rice, black gram, and skimmed milk powder, 80; rice, Bengal gram, and skimmed milk powder, 79; rice, green gram, and skimmed milk powder, 77. Lower values were obtained for the proteins of the simple mixtures of rice and pulses, as indicated in the previous paragraph. It is evident from these results that the replacement of 2 per cent of pulse proteins by an equal percentage of proteins from skimmed milk powder enhanced the biological values of the vegetable proteins in all the five cases.

The biological value of the protein mixture contained in diet No. 1, which included six vegetable foodstuffs, was high (82), being equal to that of the rice and skimmed-milk diet.

(B) EXPERIMENTS USING THE GROWTH METHOD.

In the previous section, an investigation of the supplementary values of the proteins contained in mixtures of rice with various pulses and skimmed milk

powder was reported; in these experiments the nitrogen balance-sheet method was followed and adult rats were employed. In the present experiments the biological values of the same mixtures have been studied by the growth method using young rats.

The growth method has been extensively used in determining the supplementary values of the proteins of different foodstuffs (McCollum and Simmonds, 1921; Hoagland and Snider, 1927; Kon and Markuze, 1931; Markuze, 1934; Adolph and Cheng, *loc. cit.*; Swaminathan, 1937*b*). Though numerous instances of supplementary relations between animal proteins and vegetable proteins have been discovered, only a few examples of supplementary relations between proteins of vegetable origin are known. No work has hitherto been carried out on the effect of rice and pulse proteins in supplementing each other, nor has the value of skimmed milk proteins fed in combination with rice and pulse proteins been studied by the growth method.

Experimental.

The composition of the foodstuffs and diets used was similar to those described in the first section of the paper. The technique employed was the same as that described in Part II of this series (Swaminathan, 1937*b*). The results of the growth experiments and the biological values calculated therefrom are given in Table IV. The average weekly increase in body-weight and the values of $\frac{I^2}{TP}$ are given in Tables V and VI respectively.

Discussion.

The level of protein intake in the diets was kept at approximately 8 per cent, 4 per cent of which was derived from rice and 4 per cent from the pulses or skimmed milk powder respectively or pulses and skimmed milk powder in equal quantities. The experiments were divided into two series. The diets fed to the rats in Series I contained proteins from mixtures of two foodstuffs, while the diets in Series II contained proteins from mixtures of three foodstuffs (4 per cent from rice, 2 per cent from the pulses, and 2 per cent from skimmed milk powder). In conformity with the previous investigations dealing with maintenance experiments, the replacement of 2 per cent of pulse proteins by an equal amount of milk proteins enhanced to a great extent the value of the vegetable proteins for growth.

SERIES I. *Diets containing proteins from mixtures of two foodstuffs.*—Of the six diets investigated in this series, the diet containing the proteins of rice and skimmed milk powder was found to produce the maximum growth, having a biological value of 1.86. Next in order of merit came the mixed proteins of rice and green gram, the biological value obtained being 1.29 during a period of 8 weeks. The biological values obtained for the proteins of the other four diets were as follows: rice and red gram, 1.26; rice and Bengal gram, 1.21; rice and soya bean, 1.13; and rice and black gram, 1.12.

TABLE IV.

Biological value of the mixed proteins of rice and red gram, and rice and skimmed milk powder, respectively.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
43 M	28	Rice and red gram 8.27 per cent.	59	102	43	337.1	27.88	1.54	1.55
44 M			57	88	31	228.5	18.92	1.64	
45 F			59	90	31	252.5	20.88	1.48	
43 M	56	Rice and red gram 8.27 per cent.	59	129	70	705.4	58.34	1.20	1.26
44 M			57	110	53	477.1	39.46	1.35	
45 F			59	112	53	522.3	43.19	1.23	
46 M	28	Rice and skimmed milk powder 8.41 per cent.	67	132	65	315.4	26.53	2.45	2.30
47 M			69	128	59	307.5	25.86	2.28	
48 M			61	129	68	317.0	26.66	2.55	
49 F	56	Rice and skimmed milk powder 8.41 per cent.	66	110	44	271.3	22.82	1.93	1.86
46 M			67	176	109	670.3	56.37	1.93	
47 M			69	172	103	670.4	56.38	1.83	
48 M	56	Rice and skimmed milk powder 8.41 per cent.	61	180	119	674.1	56.69	2.10	1.86
49 F			66	140	74	566.2	47.62	1.56	

TABLE IV—*contd.*
Biological value of the mixed proteins of rice and soya bean, and rice and Bengal gram, respectively.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
50 F	28	Rice and soya bean 8:42 per cent.	64	93	29	306.3	25.79	1.12	1.32
51 M			59	91	32	330.5	27.83	1.15	
52 M			65	98	33	262.0	22.06	1.50	
53 M			55	85	30	238.4	20.07	1.49	
50 F	56	Rice and soya bean 8:42 per cent.	64	114	50	600.4	50.55	0.99	1.13
51 M			59	109	50	649.3	54.67	0.92	
52 M			65	118	53	507.4	42.72	1.24	
53 M			55	110	55	480.0	40.42	1.36	
54 M	28	Rice and Bengal gram 8:19 per cent.	62	94	32	247.7	20.29	1.58	1.56
55 F			60	93	33	251.9	20.63	1.60	
56 M			55	85	30	246.5	20.19	1.49	
57 F			65	97	32	251.7	20.61	1.55	
54 M	56	Rice and Bengal gram 8:19 per cent.	62	124	62	539.4	44.18	1.40	1.21
55 F			60	105	45	537.0	43.98	1.02	
56 M			55	118	63	549.5	45.00	1.40	
57 F			65	112	47	561.4	45.98	1.02	

TABLE IV—*contd.**Biological value of the proteins of mixtures of rice and black gram, and rice and green gram, respectively.*

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
58 M	28	Rice and black gram 8.07 per cent.	50	80	30	204.4	16.49	1.82	1.53
59 F			62	84	22	223.4	18.03	1.22	
60 M			59	87	28	247.8	19.98	1.40	
61 F			69	107	38	286.3	23.11	1.65	
58 M	56	Rice and black gram 8.07 per cent.	50	100	50	457.6	37.92	1.32	1.12
59 F			62	104	42	510.9	41.23	1.02	
60 M			59	104	45	519.1	41.89	1.07	
61 F			69	118	49	560.9	45.26	1.08	
62 M	28	Rice and green gram 8.20 per cent.	62	94	32	255.3	20.93	1.53	1.60
63 M			62	93	31	262.9	21.56	1.44	
64 M			62	102	40	257.0	21.07	1.95	
65 F			64	93	29	241.1	19.77	1.47	
62 M	56	Rice and green gram 8.20 per cent.	62	124	62	565.7	46.39	1.34	1.29
63 M			62	127	65	591.2	48.48	1.34	
64 M			62	125	63	573.4	47.02	1.34	
65 F			64	114	50	537.9	44.11	1.13	

TABLE IV—*contd.*

Biological value of the proteins of mixtures of rice, red gram and skimmed milk powder, and rice, soya bean and skimmed milk powder, respectively.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
66 M	28	Rice, red gram and skimmed milk powder 8·10 per cent.	52	90	38	253·8	20·56	1·83	1·76
67 F			57	91	34	255·1	20·66	1·65	
68 M			57	92	35	261·6	21·20	1·65	
69 F			60	100	40	257·5	20·86	1·92	
66 M	56	Rice, red gram and skimmed milk powder 8·10 per cent.	52	124	72	530·5	42·97	1·68	1·49
67 F			57	120	63	550·8	44·61	1·41	
68 M			57	128	71	566·5	45·89	1·55	
69 F			60	120	60	558·4	45·23	1·33	
70 M	28	Rice, soya bean and skimmed milk powder 8·19 per cent.	55	88	33	243·9	19·98	1·66	1·58
71 F			60	88	28	260·2	21·31	1·31	
72 M			65	105	40	275·5	22·56	1·77	
70 M	56	Rice, soya bean and skimmed milk powder 8·19 per cent.	55	115	60	532·0	43·57	1·39	1·33
71 F			60	115	55	534·9	43·81	1·26	
72 M			65	125	60	551·8	44·19	1·36	

TABLE IV—contd.

Biological value of the proteins of mixtures of rice, black gram and skimmed milk powder, and rice, Bengal gram and skimmed milk powder, respectively.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
73 M	28	Rice, black gram and skimmed milk powder 8.06 per cent.	59	90	31	237.7	19.16	1.62	1.74
74 F			64	102	38	282.0	22.73	1.67	
75 M			64	107	43	286.1	23.06	1.86	
76 F			65	112	47	324.9	26.19	1.79	
73 M	56	Rice, black gram and skimmed milk powder 8.06 per cent.	59	120	61	524.0	42.23	1.44	1.39
74 F			64	117	53	567.2	45.72	1.16	
75 M			64	140	76	595.4	45.99	1.58	
76 F			65	137	72	641.7	51.72	1.39	
77 M	28	Rice, Bengal gram and skimmed milk powder 8.30 per cent.	55	110	55	246.7	20.47	2.69	2.25
78 M			54	105	51	233.0	19.34	2.64	
79 F			59	94	35	233.3	19.36	1.81	
80 F			59	94	35	229.0	19.03	1.84	
77 M	56	Rice, Bengal gram and skimmed milk powder 8.30 per cent.	55	158	103	553.5	45.94	2.24	1.89
78 M			54	142	88	509.5	42.29	2.08	
79 F			59	123	64	471.4	39.13	1.64	
80 F			59	121	62	472.3	39.20	1.58	

TABLE IV—*concd.*
Biological value of the proteins of a mixture of rice, green gram and skimmed milk powder.

Rat number and sex.	Duration of experimental period, days.	Source and percentage of protein in the diet.	Initial body-weight, g.	Final body-weight, g.	Gain in body-weight, g.	Food intake, g.	Protein intake, g.	Biological value gain in body-weight, g. protein intake, g.	Average biological value.
81 M	28	Rice, green gram and skimmed milk powder 8.29 per cent.	58	135	77	277.3	22.99	3.35	2.47
82 M			58	115	57	256.3	21.24	2.68	
83 F			58	97	39	225.0	18.65	2.09	
84 F			60	97	37	226.3	18.76	1.97	
81 M	56	Rice, green gram and skimmed milk powder 8.29 per cent.	58	175	117	580.0	48.08	2.43	2.04
82 M			58	157	99	526.4	43.64	2.27	
83 F			58	125	67	456.6	37.86	1.77	
84 F			60	127	67	480.3	39.89	1.68	

TABLE V.

*Average weekly increase in body-weight during a period of 8 weeks
at an 8 per cent level of protein intake.*

Sources of protein in diet.	Average weekly increase in body-weight, g.
SERIES 1.	
Raw milled rice and skimmed milk powder ..	12·6
.. .. green gram ..	7·5
.. .. red gram ..	7·4
.. .. Bengal gram ..	6·8
.. .. soya bean ..	6·5
.. .. black gram ..	5·9
SERIES 2.	
Raw milled rice, green gram and skimmed milk powder.	10·9
.. .. Bengal gram and skimmed milk powder.	9·9
.. .. red gram and skimmed milk powder.	8·3
.. .. black gram and skimmed milk powder.	8·3
.. .. soya bean and skimmed milk powder.	7·3

TABLE VI.

Average daily increase in body-weight ($\frac{I}{T}$), biological value ($\frac{I}{P}$) and the values of ($\frac{I^2}{TP}$) at an 8 per cent level of protein intake.

Sources of protein in the diet.	AVERAGE DAILY INCREASE IN BODY-WEIGHT ($\frac{I}{T}$).		AVERAGE BIOLOGICAL VALUE ($\frac{I}{P}$).		AVERAGE VALUE OF ($\frac{I^2}{TP}$).	
	28 days.	56 days.	28 days.	56 days.	28 days.	56 days.
Raw milled rice and red gram ..	1.26	1.06	1.55	1.26	1.92	1.34
" " " skinned milk powder	2.11	1.81	2.30	1.86	4.85	3.37
" " " soya bean ..	1.11	0.93	1.32	1.13	1.47	1.05
" " " Bengal gram ..	1.13	0.97	1.56	1.21	1.76	1.17
" " " black gram ..	1.05	0.83	1.53	1.12	1.61	0.93
" " " green gram ..	1.18	1.07	1.60	1.29	1.89	1.38
" " " red gram, and skimmed milk powder.	1.31	1.19	1.76	1.49	2.31	1.77
" " " soya bean, and skimmed milk powder.	1.20	1.04	1.58	1.33	1.90	1.38
" " " black gram, and skimmed milk powder.	1.42	1.17	1.74	1.39	2.47	1.63
" " " Bengal gram, and skimmed milk powder.	1.57	1.42	2.25	1.89	3.53	2.08
" " " green gram, and skimmed milk powder.	1.89	1.56	2.47	2.04	4.67	3.18

SERIES II. *Diets containing proteins from mixtures of three foodstuffs.*—Of the five diets investigated under this series, the mixed proteins of rice, green gram, and skimmed milk powder were found to be the best, having a biological value of 2.04, while the value obtained for the proteins of rice and green gram was only 1.29; hence it is evident that milk proteins form an excellent supplement to the proteins of rice and green gram. The biological values of the mixed proteins of the other four diets investigated were as follows: rice, Bengal gram, and skimmed milk powder, 1.89; rice, red gram, and skimmed milk powder, 1.49; rice, black gram, and skimmed milk powder, 1.39; and rice, soya bean, and skimmed milk powder, 1.33. These figures are higher than those obtained for the proteins of mixtures of rice and pulses.

Increase in body-weight and biological value.

The average weekly increase in body-weight during a period of 8 weeks with the different diets is shown in Table V. The greatest increase (12.6 g.) was obtained with the mixed proteins of rice and skimmed milk powder, and the smallest (5.9 g.) with the mixed proteins of rice and black gram. Taking weight increase as the sole criterion of biological value, the mixed proteins investigated in these experiments would range themselves as follows in descending order of merit: *Series I*: rice and skimmed milk powder; rice and green gram; rice and red gram; rice and Bengal gram; rice and soya bean; and rice and black gram; *Series II*: rice, green gram, and skimmed milk powder; rice, Bengal gram, and skimmed milk powder; rice, red gram, and skimmed milk powder; rice, black gram, and skimmed milk powder; and rice, soya bean, and skimmed milk powder. The conclusions so arrived at are in agreement with the biological values assessed by the standard growth method based on the relation between increase in weight and protein intake. The growth curves are shown in Figs. 1 and 2.

Biological values and the values of $\frac{I^2}{TP}$.

The value of $\frac{I^2}{TP}$ may represent more completely the relative values of different proteins in nutrition than the biological values obtained by the growth method, since it is a product of the biological value $\frac{I}{P}$ and the average daily increase in body-weight $\frac{I}{T}$. The values of $\frac{I^2}{TP}$ have been calculated for the two periods of first 4 weeks and 8 weeks respectively, and are shown in Table VII along with the corresponding biological values and the average daily increase in body-weight. The average values of $\frac{I^2}{TP}$ obtained during 8 weeks are in general correspondence with the biological values.

Supplementary values of proteins for maintenance versus growth.—In Part II of this series (Swaminathan, 1937b) the relative protein requirements for maintenance versus growth were discussed. The results obtained in the earlier papers (Parts I and II) with the proteins of single foodstuffs were in general in accordance with

the views expressed by Sherman (1933) that 'it is quite reasonable to suppose that proteins of different efficiency for growth may show much more nearly equal efficiency in the normal maintenance nutrition of adults; though it is also true,

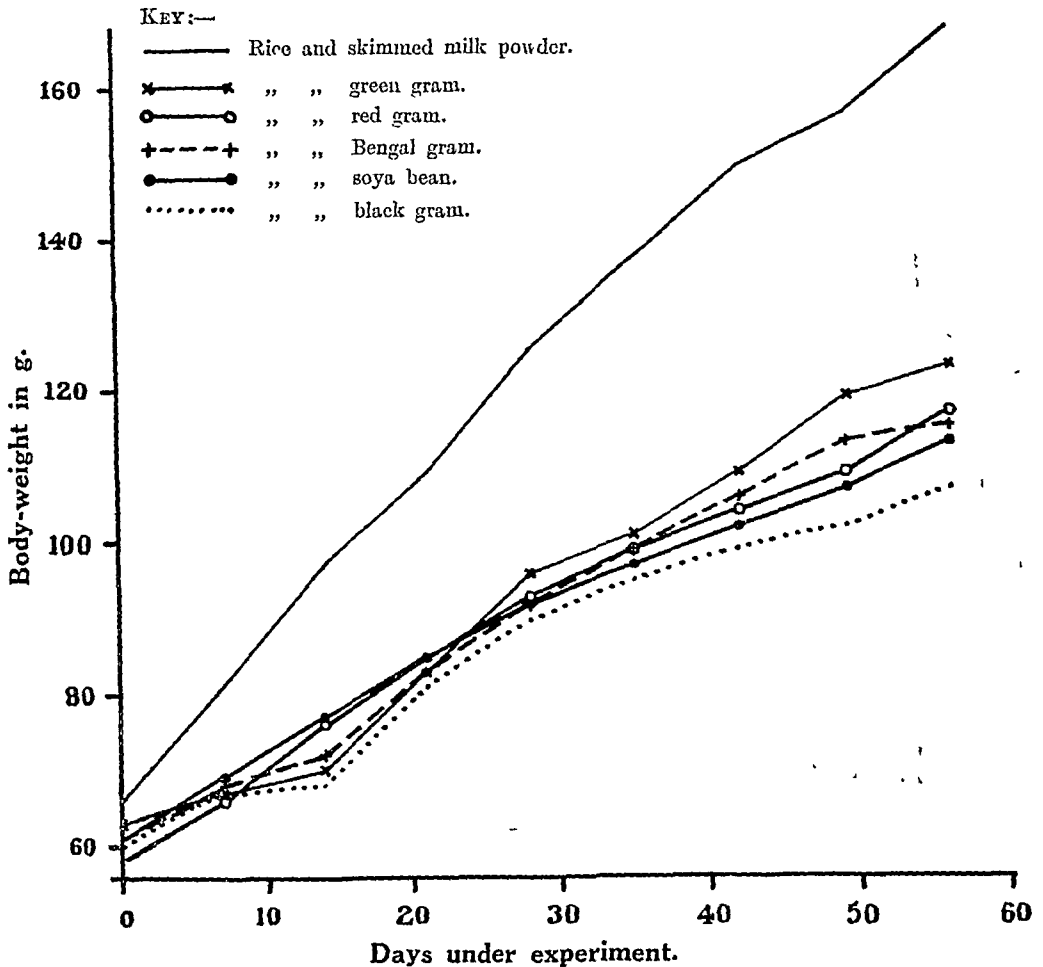


FIG. 1. Showing average increase in weight of groups of rats receiving proteins from mixtures of two foodstuffs.

so far as is known, that the proteins more efficient for growth are likewise more efficient for maintenance'.

The protein mixture investigated may be broadly divided into three groups as regards the biological values (Table VII). *Group I*: containing proteins which

have a high efficiency for maintenance as for growth, the important examples being the following : (1) rice and skimmed milk powder ; (2) rice, red gram, and skimmed milk powder ; (3) rice and red gram ; (4) rice, soya bean, and skimmed milk powder ; (5) rice, black gram, and skimmed milk powder ; and (6) rice, Bengal gram, and skimmed milk powder ; *Group II* : containing proteins more efficient

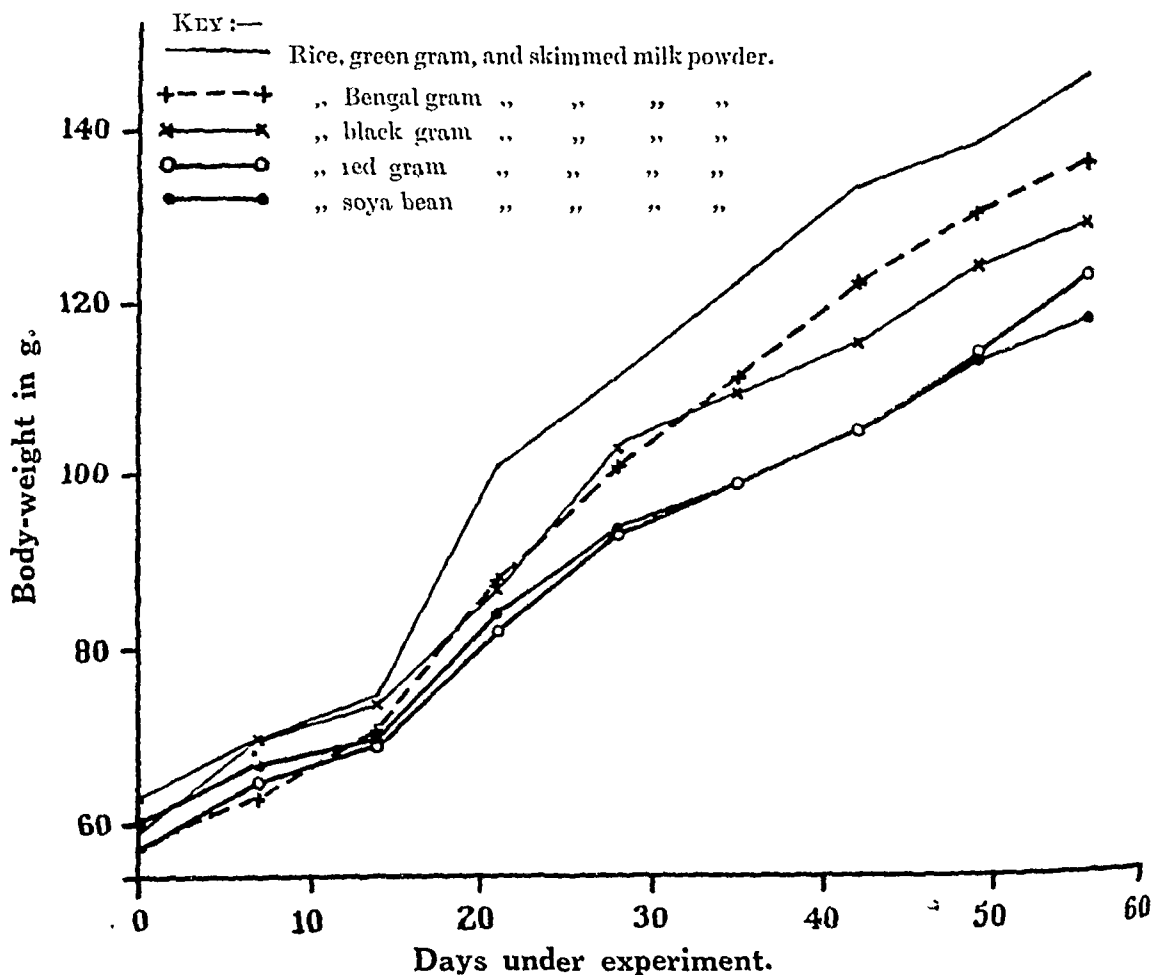


FIG. 2. Showing average increase in weight of groups of rats receiving proteins from mixtures of three foodstuffs.

for growth than for maintenance. examples being the following : (1) rice and green gram ; (2) rice, green gram, and skimmed milk powder ; and *Group III* : containing proteins more efficient for maintenance than for growth, including the following : (1) rice and soya bean ; (2) rice and Bengal gram ; and (3) rice and black gram.

TABLE VII.

Values for maintenance and for 'growth and maintenance' compared.

Sources of protein in the diets.	Biological value for maintenance measured by the nitrogen balance method (per cent).	Biological value for growth and maintenance measured by the growth method during 8 weeks.
SERIES 1.		
Raw milled rice and skimmed milk powder ..	80	1.86
„ „ „ „ red gram	76	1.26
„ „ „ „ soya bean	68	1.13
„ „ „ „ Bengal gram	66	1.21
„ „ „ „ black gram	61	1.12
„ „ „ „ green gram	59	1.29
SERIES 2.		
Raw milled rice, red gram, and skimmed milk powder.	90	1.49
„ „ „ soya bean, and skimmed milk powder.	86	1.33
„ „ „ black gram, and skimmed milk powder.	80	1.39
„ „ „ Bengal gram, and skimmed milk powder.	79	1.89
„ „ „ green gram, and skimmed milk powder.	77	2.04

CONCLUSIONS.

In so far as tests of biological values, concerned with the maintenance of nitrogenous equilibrium in adult rats and the growth and maintenance of young rats, are applicable to human nutrition, the following conclusions of practical importance may be suggested :—

Large quantities of animal proteins (e.g., milk proteins) are not required to raise the biological value of the proteins of a diet based on rice and other vegetable foods. Small amounts are of supplementary value.

The mixture of proteins contained in a suitable diet of solely vegetable origin may be of high biological value.

SUMMARY.

Section A.

(1) The biological values and digestibility co-efficients of the proteins from mixtures of rice, pulses, and skimmed milk powder have been determined by the nitrogen balance-sheet method for 'maintenance' in adult rats at an 8 per cent level of protein intake. The figures obtained with mixtures of two proteins were as follows: rice and skimmed milk powder, 80 and 89; rice and red gram, 76 and 81; rice and soya bean, 68 and 84; rice and Bengal gram, 66 and 85; rice and black gram, 61 and 82; and rice and green gram, 56 and 90, respectively.

(2) The same values for the proteins of mixtures of three foodstuffs were as follows: rice, red gram, and skimmed milk, 90 and 87; rice, soya bean, and skimmed milk, 86 and 93; rice, black gram, and skimmed milk, 80 and 92; rice, Bengal gram, and skimmed milk, 79 and 96; rice, green gram, and skimmed milk, 77 and 95, respectively.

(3) At a 10 per cent level of protein intake, the biological value and the digestibility co-efficient of the proteins of a mixed vegetarian diet (rice, red gram, black gram, amaranth leaves, brinjal, and coco-nut) were found to be 82 and 92, respectively.

Section B.

The relative supplementary values of the mixed proteins of rice, certain pulses, and skimmed milk powder for 'growth and maintenance' have been studied by the growth method using young rats. The values obtained at an 8 per cent level of protein intake during a period of 8 weeks were as follows: (a) mixtures of two foodstuffs: rice and skimmed milk, 1.86; rice and green gram, 1.29; rice and red gram, 1.26; rice and Bengal gram, 1.21; rice and soya bean, 1.13; rice and black gram, 1.12; (b) mixtures of three foodstuffs: rice, green gram, and skimmed milk, 2.04; rice, Bengal gram, and skimmed milk, 1.89; rice, red gram, and skimmed milk, 1.49; rice, black gram, and skimmed milk, 1.39; rice, soya bean, and skimmed milk, 1.33.

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RELATIVE DIGESTIBILITIES OF EDIBLE FATS BY CASTOR-SEED AND PANCREATIC LIPASES.

BY

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FATS form an important source of energy in the diet and they are more effective for this purpose than either proteins or carbohydrates. The importance of their dietetic value has received much less attention at the hands of the investigators than other food constituents and is consequently less well understood. It is generally assumed that fats are normally well assimilated, and that they do not vary much in this respect to affect materially the amount of energy which the body receives from them. It has also been suggested that thoroughness of digestion of fats is related to their melting points, those having melting points above the body temperature being not quite so thoroughly assimilable (Langworthy, 1923). The digestibility co-efficients of hydrogenated oils of different melting points are, according to Holmes and Deuel (1921), inversely proportional to the degree of hardness. Recently, contrary to the findings of Langworthy (*loc. cit.*), Steenbock *et al.* (1936) have shown from *in vivo* experiments that there exists a real difference in the absorption rates of various vegetable and animal fats.

In India butter-fat is very extensively used as an article of food. The vegetable oils like sesame, ground-nut, mustard, mahua, or coco-nut are also much in use for edible purposes throughout India. Indeed, the vegetarian population in India has no other source of material for the supply of fat except ghee (clarified butter from cow, buffalo, or goat) and vegetable oils.

Because of the wide differences in chemical composition and physical characteristics of the various fats, the question naturally arises regarding their relative digestibility. Godbole and Sadgopal (1930) have classified fats into four groups depending on their digestibility and absorbability by the system, viz., (i) butter-fat; (ii) coco-nut oil; (iii) other oils rich in oleic glycerides such as sesame and safflower oils; and (iv) animal fats and hydrogenated oils of vegetable and animal origin.

In the present investigation a study of the relative digestibilities of the edible fats has been made using lipases from vegetable (castor seed) and animal (pancreatic) sources, and simulating as far as possible the body conditions of temperature, H-ion concentrations, etc. It was thought that if wide differences in the hydrolysis of butter-fat on the one hand and other oils on the other were obtained, a method could be evolved for the detection of adulteration of ghee with other fats by the enzymic hydrolysis method. The results presented here may prove of considerable value in throwing light on the complexity of the problem and in explaining some of the conflicting results of previous workers. Since the experimental techniques employed with castor-seed and pancreatic lipases vary greatly, the experimental portion has been, for convenience, divided into two parts.

EXPERIMENTAL.

PART I. Digestion of fats by castor-seed lipase.

The fats selected for the investigation were butter-fat (both cow and buffalo), coco-nut, sesame, and ground-nut oils. The oils were prepared in the laboratory from cleansed seeds by crushing. The oils were then neutralized, soaps washed off, and the suspended impurities removed by filtering through Fuller's earth.

The enzyme material was prepared from a good variety of castor beans (*Ricinus communis*) by the method outlined by Longnecker and Haley (1935).

1. *Determination of activity.*—For the determination of activity, a series of test-tubes ($6" \times \frac{3}{4}"$) was set up each containing 2.0 g. of the fat under test, 0.1 g. of the active lipase material, and three drops of toluene to prevent bacterial action. The hydrolysis was started by the addition of 1.0 c.c. of M-5 acetic acid-sodium acetate buffer, which acts as the activator for the castor-seed lipase. As soon as the buffer was added, the reaction mixture was emulsified by vigorous shaking for exactly three minutes. The tubes were then kept in a thermostat maintained at $35^{\circ} \pm 0.1^{\circ}\text{C}$. Suitable blanks were always run along with the reaction mixtures. After a definite period of hydrolysis the reaction mixture was poured into 50 c.c. of hot 95 per cent alcohol which served to check the enzymic action and the free fatty acids in the mixture, produced as a result of hydrolysis, were titrated with 0.1 N alkali using phenolphthalein as indicator.

The extent of digestion was measured in terms of c.c. of 0.1 N alkali required to neutralize the fatty acids formed after a known interval. The results in the following tables represent averages of duplicate determinations which generally agreed closely. The starting time for each experiment was the time of addition of the buffer to the reaction mixture.

2. *Effect of pH on the hydrolysis of fats.*—The optimum pH for the hydrolysis by castor-bean lipase has been found by Haley and Lyman (1921) to lie at pH 4.7 to 4.8, while Guillemet (1931) stated that the value for the optimal pH depends on the nature of the buffer employed. The latter author suggested that the amount of albuminous matter accompanying the lipase plays an important rôle.

Since the object of the present investigation was to compare the digestibility of butter-fat with other oils, it was considered necessary to determine the optimum

pH for the digestion of butter-fat using acetic acid-sodium acetate buffer to control the pH of the reaction mixture. The results are presented in Table I :—

TABLE I.

Relation between H-ion concentration and digestion of butter-fat by castor-seed lipase.

pH :—	3.2	3.4	3.7	4.2	4.4	4.6	4.8	5.0
	c.c. of 0.1 N alkali required to neutralize fatty acids.							
30 minutes' hydrolysis .	15.70	15.50	16.40	18.15	19.1	14.95	2.65	0.60
60 minutes' hydrolysis .	17.85	19.15	20.05	22.25	22.4	17.90	8.35	0.95

The results show that the optimum pH lies between 4.2 and 4.4. In the subsequent determinations of digestion the pH was therefore always adjusted to 4.2.

3. *Relative rates of digestion of fats.*—The results of the relative digestibilities of ghee and other edible oils are represented graphically in Fig. 1.

It is seen that there is a marked difference in the degree of digestibility of the different fats, the order of the ease with which the fats are decomposed by castor-bean lipase being butter-fat, coco-nut oil, sesame oil and ground-nut oil, the last two oils being digested almost to the same extent.

An observation of interest is that the course of the hydrolysis of butter-fat is quite different from those of other oils. The course of butter-fat hydrolysis with time is linear till a considerable portion of the fat has been hydrolysed, while in case of the other oils the rate of hydrolysis slows down even from the beginning. This peculiar difference in the rates of hydrolysis of butter-fat and other oils may be ascribed to difference in their chemical composition. It is well known that butter-fat differs essentially in composition from all other oils and fats in having a large percentage of steam-volatile and water-soluble fatty acids. It is therefore likely that the difference in the digestibility of ghee and other oils may be due to the differences in the specificity of castor-seed lipase for the water-soluble and steam-volatile fatty acids on the one hand and insoluble fatty acids on the other. The difference in the digestion curves may also perhaps be accounted for by a true equilibrium with the synthetic reaction. This would probably mean that in the hydrolysis of butter-fat, the synthetic reaction is not so pronounced as it is in the case of other oils. This suggestion is borne out by the observation of Velluz (1934) who, investigating the synthesis of esters by the lipase of castor beans, found that when the lipase was added to equimolecular mixtures of alcohol and fatty acids and incubated at 37°C. no saponification occurred with any acids containing less than seven carbon atoms in a straight chain. Thus, butyric and

physical state of the enzyme and associated substances, (b) the pH of the medium, and (c) the proper emulsification of the digestion mixture. A rigorous control of these factors is, therefore, necessary in order to obtain accurate and readily reproducible experimental results. In the present investigation an attempt has been made to control all these factors, by simulating, as far as possible, the optimum body conditions of temperature, H-ion concentration and fat emulsification. The primary purpose was, therefore, (i) to determine the optimum conditions for the digestion of each individual fat by pancreatic lipase; (ii) to study the relative digestibility of the fats under the optimum conditions so obtained; and (iii) to investigate the function of the bile salts on the digestion of the various fats.

The pancreatic lipase was prepared from fresh pig pancreas. The adhering fat was removed as far as possible and the pancreas macerated well in a mortar. The pancreas was then treated with acetone, acetone-ether, and finally with ether in a manner similar to that described by Willstatter and Waldschmidt-Leitz (1923). The fat-free product was ground to a fine powder and kept in a desiccator. For preparing the extract 10 g. of the powder was triturated with 60 c.c. of 85 per cent aqueous glycerol, and shaken for 4 hours at room temperature (26°C.). The coarse particles were removed by centrifuging, and the slightly turbid extract was directly used for the experiments.

5. *Determination of activity.*—The digestion was carried out as follows: A series of test-tubes was set up each containing 2.0 g. of fat and 1 c.c. of the buffer. Other substances whose effect on the digestion of fats was to be tried were added to this mixture. Finally, 1 c.c. of the enzymic extract was added and immediately shaken with the hand for 3 minutes, and kept in a thermostat at $35^{\circ} \pm 0.1^{\circ}\text{C}$. The tubes were removed for titration at known intervals, and the contents quickly transferred to flasks containing 50 c.c. of hot 95 per cent alcohol and 10 c.c. of ethyl ether added (*cf.* Willstatter and Waldschmidt-Leitz, *loc. cit.*). The mixture was titrated against 0.1 N alkali using phenolphthalein as indicator. The titration value of a control mixture containing boiled enzyme but otherwise identical with the active digest was taken to represent the zero point. The results given are the mean of duplicate determinations, corrected for corresponding controls, and are expressed in c.c. of 0.1 N alkali required to neutralize the fatty acids liberated in the reaction mixture after a definite period of digestion.

6. *Influence of H-ion concentration.*—The optimum pH for the action of pancreatic lipase has been determined by several workers and widely varying values have been obtained. Thus, Davidsohn (1912) found the value for optimum pH to be 8.0 and in a later communication (1913) he fixed the value at 8.5 as the optimum pH for pancreatic lipase. Rona and Bien (1914) have shown that there is an optimum zone over which the lipase of pancreatic extract is most active, namely from pH 8.4 to pH 9.0. According to Umeda (1915) the optimum pH for the action of lipase on olive oil in phosphate buffer lies at about 7.4. Rona and Pavlovic (1923) obtained for the optimum pH a value of 6.95 to 8.0, when a saline extract of pancreas was used for activity determination. Willstatter and Waldschmidt-Leitz (*loc. cit.*) in their researches on lipase have employed ammonia-ammonium chloride buffer at pH 9.2 when using olive oil as the substrate, and a similar buffer at pH 8.6 when tributyrin was the substrate. Anrep, Lush and Palmer (1925) have determined lipase activity in phosphate buffer solution at pH 7.8. Platt and Dawson (1925)

have shown that with a purified pancreatic powder containing lipase, the optimum pH in phosphate and in phosphate-borax buffer solutions was 7.0 and in borax-boric mixture there was an optimum zone near about 8.4.

It would appear from the foregoing that the pH-activity relations depend on several factors, such as (a) the nature of the substrate, (b) the buffer salts used, and (c) the presence of the associated substances. In the following experiments a detailed investigation of the influence of these factors on the relationship between pH and lipase activity has been conducted and the results reported throw fresh light on the nature of the lipase and also on the process of digestion of fats in the animal body.

(i) *Influence of ammonia-ammonium chloride (N-10) buffer on optimum pH.*—The reaction mixture consisted of 2.0 g. of fat, 1 c.c. of buffer, 1 c.c. of lipase, 0.5 c.c. of egg albumen (1 per cent solution). Readings were taken after 30 minutes' interval. The results are given in Table III:—

TABLE III.

Influence of ammonia-ammonium chloride buffer on optimum pH.

pH:—	8.0	8.3	8.6	8.9	9.2	9.5	9.8	10.1	11.0
	c.c. of 0.1 N alkali required to neutralize fatty acids.								
Cow ghee	5.0	5.5	5.6	6.0	8.5	8.5	10.7	12.6	..
Buffalo ghee ..	3.9	4.7	5.2	6.4	7.3	8.6	8.9	9.3	10.4
Sesame oil ..	3.9	3.1	3.9	6.9	10.0	13.7	11.8	11.8	..
Ground-nut oil	2.7	3.0	4.6	7.9	11.9	14.1	14.6	13.1	..

It is seen from these results that the optimal activity of pancreatic lipase in ammonia-ammonium chloride buffer lies at about pH 9.5 for sesame and ground-nut oils, whilst for the digestion of butter-fat there appears to be no definite pH optimum.

Some preliminary experiments on the effect of addition of egg albumen, calcium chloride, egg albumen and calcium chloride, sodium taurocholate, etc., to ground-nut oil showed that addition of these substances brings about an increase in the rate of digestion without altering the pH activity relationship, the pH optima always occurring at 9.5.

(ii) *Influence of glycine buffer (N-10) on pH optima.*—In the following experiments, glycine buffer (N-10) of different pH values was employed in place of the ammonia buffer (Table IV):—

TABLE IV.

Influence of glycine buffer.

pH:—	8.5	9.3	10.1	11.0	11.5	12.0	12.6	12.9	13.0
	c.c. of 0.1 N alkali required to neutralize fatty acids.								
Cow ghee ..	3.9	5.6	4.6	5.2	5.7	6.2	7.4	3.2	..
Sesame oil ..	5.0	2.8	3.3	8.3	8.6	11.5	12.9	9.7	2.6
Ground-nut oil	7.8	4.4	2.9	8.8	7.9	11.0	9.9	7.2	5.0
Coco-nut oil ..	4.7	6.9	9.5	10.7	9.8	9.7	10.2	12.0	6.2

It will be seen from the figures that the general type of pH-activity curve for the digestion of fats shows two maxima, one between pH 8.5 and pH 10.0, and the other between pH 12.0 and pH 13.0. This peculiar behaviour is exhibited by all the fats studied. The nature of these pH-activity curves is, however, altered when calcium chloride and egg albumen are added to the digestion mixture. This is indicated in the results given in Table V. The reaction mixture consisted of 2.0 g. of fat, 1 c.c. lipase extract, 1 c.c. glycine buffer, 0.5 c.c. of 0.6 per cent calcium chloride and 0.5 c.c. of 1.0 per cent egg albumen solution.

TABLE V.

Optimum pH of fats in presence of activators and with glycine buffer.

pH:—	8.5	9.3	10.1	11.0	11.5	12.0	12.6	12.9	13.0
	c.c. of 0.1 N alkali required to neutralize fatty acids.								
Cow ghee ..	5.4	7.5	9.6	9.7	10.2	10.8	10.4	11.5	11.8
Sesame oil ..	3.0	8.2	13.0	13.7	13.7	12.5	7.2	9.1	..
Ground-nut oil	5.1	9.0	11.7	10.6	10.6	13.5	12.7	9.9	7.9
Coco-nut oil ..	5.0	7.5	12.1	12.4	12.0	12.6	13.0	13.3	3.6

Thus, the shape of the pH-activity curve depends not only on the nature of the buffer employed but also on the presence of certain substances associated with the enzyme.

7. *Relative digestibility of fats by pancreatic lipase.*—Figs. 2 and 3 indicate the digestion curves of fats by pancreatic lipase. The digestion measurements

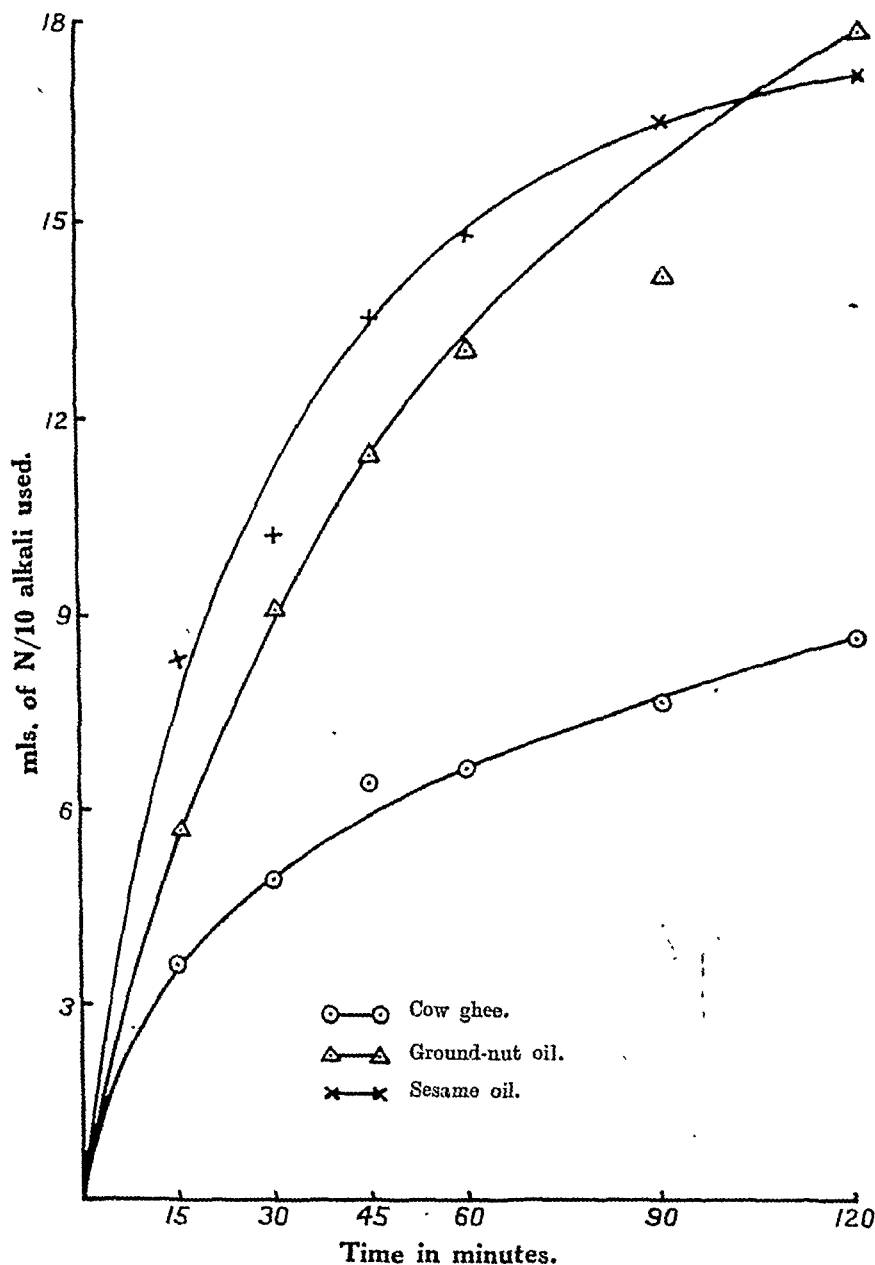


FIG. 2. Relative digestibility of oils by pancreatic lipase at pH 12.6.

were carried out as described before at the two pH optima, 12.6 and 9.6, using glycine buffer. In both cases the reaction mixture consisted of 2.0 g. fat, 1 c.c. buffer, and 1 c.c. of the lipase extract.

It would be seen that butter-fat and coco-nut oil are more easily digested at pH 9.3 than the other fats studied. The digestions of the fats at pH 12.6 follow

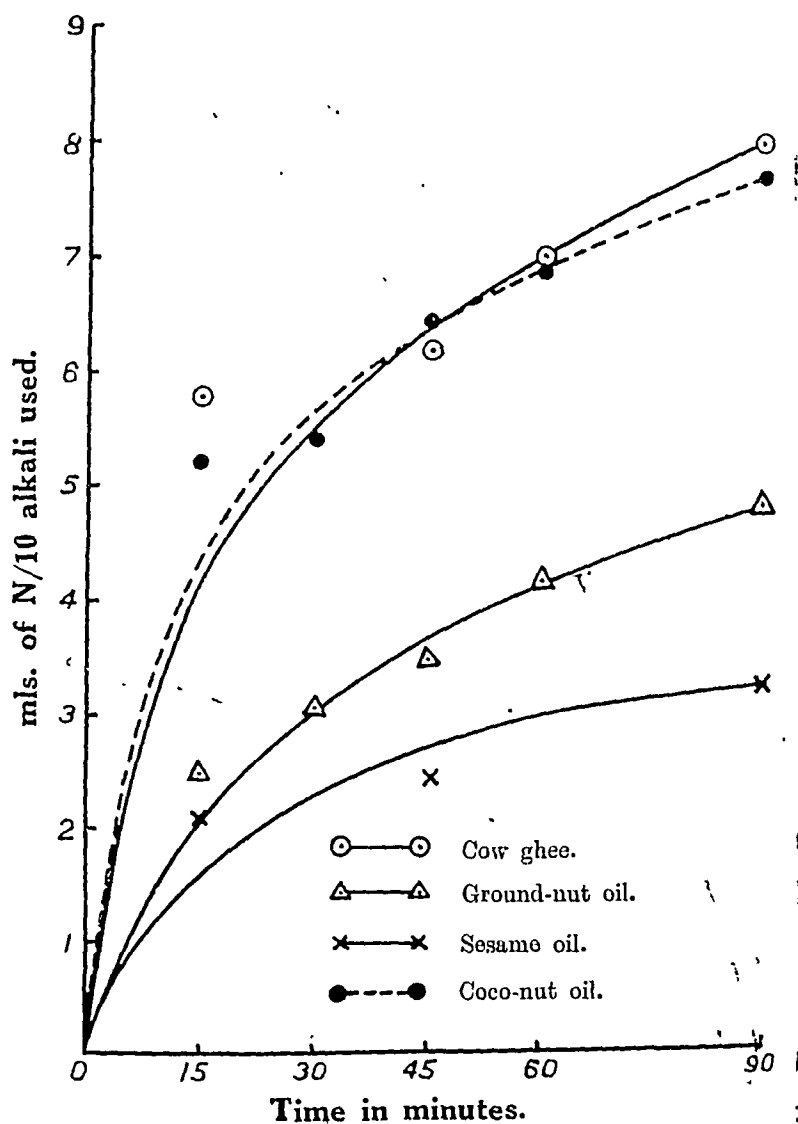


FIG. 3. Relative digestibility of oils by pancreatic lipase at pH 9.3.

an entirely different course. At this more alkaline pH, ghee is less digestible than the other oils. It would appear, therefore, that the H-ion concentration of the digestion mixture is an important factor in controlling the digestion of different fats.

8. *Effect of bile salt on the digestion of fat by pancreatic lipase.*—The accelerating action of bile salts and blood serum on pancreatic lipase has been observed by many workers (Pottevin, 1903; Terroine, 1914; Hamsik, 1911; Mellanby and Woolley, 1914; and other workers). Bile salts and serum, according to Shaw-Mackenzie (1911, 1912, 1914) and Rosenheim (1910), act as co-enzymes. They also found that a number of different compounds of varying structure can exert an augmentative action on lipase, for example, alcohol (in small quantities), sodium oleate, cholate and glycocholate, saponin and digitonin. Cholesterol, on the other hand, had a marked diminishing action on the lipolytic augmentation of serum and bile salts.

The researches of Willstatter *et al.* (1923, 1924) have shown that calcium chloride, bile salts, and egg albumen activate pancreatic lipase in alkaline medium and inhibit it in an acid medium. In the case of the bile salts it was shown that either gastric or pancreatic lipase could be activated only after a certain degree of purification of the enzyme had been attained (Willstatter and Bamann, 1928). At higher concentrations the bile salts showed decreased activation (Willstatter and Memmen, 1923).

It has also been reported that the degree of dispersion of the substrate has very little influence on the rate of lipase action (Rona and Kleinmann, 1926), and that bile salts activate the hydrolysis of soluble esters by lipase (Terroine, 1910). In contrast, the work of other investigators has indicated that bile salts inhibit liver esterase (Wishart, 1920).

It would thus appear that the accelerating effect of the bile salts on digestion by pancreatic lipase would depend on a number of factors such as the pH of the digestion mixture, the source of the lipase, and also on the degree of purity of the enzyme. The results of the present study have introduced another factor, namely, the nature of the substrate, which greatly influences the accelerating effect of bile salts on the lipase digestion.

(i) *Effect of sodium taurocholate concentration.*—The bile salt used for the present study was Merck's pure sodium taurocholate ($C_{26}H_{44}O_7HSNa$).

Two grammes of butter-fat were added to each of the test-tubes containing 1 c.c. of glycine buffer (pH 9.3). With the first for a blank, increasing quantities of taurocholate solution were added to the others, then 1 c.c. of lipase extract was added to each test-tube, shaken for 3 minutes and kept at 35°C. for 30 minutes. The subsequent procedure was the same as described before. The results are given in Table VI:—

TABLE VI.

Effect of concentration of sodium taurocholate on the digestion of butter-fat.

Concentration of sodium taurocholate:—	Control.	M-100,000.	M-10,000.	M-1,000.	M-100.
	c.c. of 0.1 N alkali required to neutralize fatty acids.				
Activity in c.c. of 0.1 N alkali	3.6	7.0	7.7	6.4	5.9

It is found that sodium taurocholate in concentrations of M-100,000 and M-10,000 considerably accelerates the digestion of butter-fat, while at higher concentrations the accelerating effect tends to decrease.

(ii) *Effect of bile salt on the digestion of fats by pancreatic lipase.*—A comparison of the effect of different fats as substrates on the accelerating effect of taurocholate (M-10,000) is given in Table VII. The readings were taken at 30 minutes' interval:—

TABLE VII.

Effect of bile salt.

	Cow ghec.	Sesame oil.	Ground-nut oil.	Coco-nut oil.
	c.c. of 0.1 N alkali required to neutralize fatty acids.			
Without taurocholate	5.6	1.8	2.4	5.4
With taurocholate ..	7.5	2.0	2.4	7.6

The results show that bile does not always augment the digestion of fats by pancreatic lipase. The accelerating effect depends on the nature of the fat used as substrate. Thus, practically, no effect was observed on the digestion of sesame and ground-nut oils; whilst the digestion of butter-fat and coco-nut oil is considerably augmented by the salt. Since the accelerating effect of the bile salt is different with different fats, it is suggested that in order to be effective the accelerating agent must react with the enzyme as well as the substrate.

(iii) *Relative digestibilities of fats in presence of sodium taurocholate.*—With a view to studying the digestibility of the fats by simulating body conditions of fat emulsification, the following experiments were conducted on the relative digestibilities of the fats in presence of optimum concentration of the bile salt. The results are presented graphically in Fig. 4.

It can be seen from the figure that butter-fat and coco-nut oil are more easily digested in presence of bile salts than sesame and ground-nut oils. Further, butter-fat requires a comparatively much shorter period to reach the equilibrium point

than the other fats. Fats may be therefore arranged according to the decreasing magnitude of their digestibility as follows: butter-fat, coco-nut oil, ground-nut oil, and sesame oil.

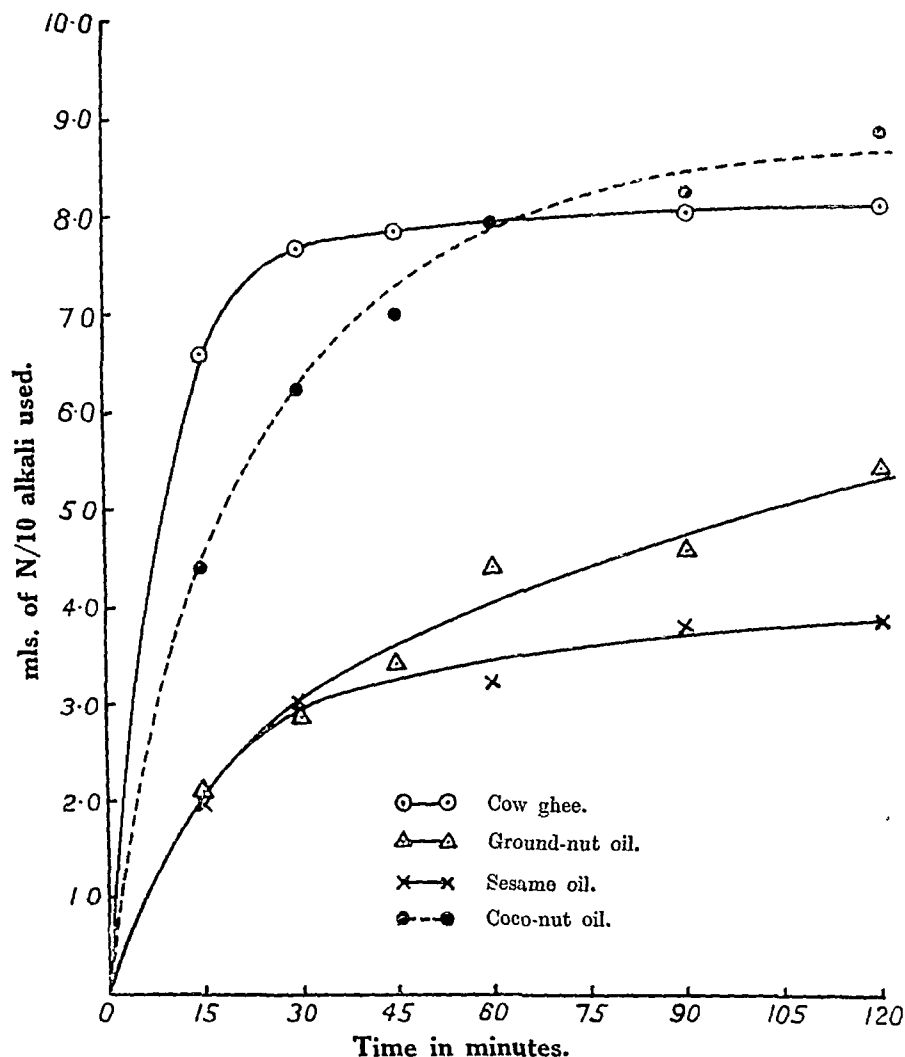


FIG. 4. Relative digestibility of oils by pancreatic lipase at pH 9.3 in presence of M-10,000 sodium taurocholate.

DISCUSSION.

Conflicting views have been held by previous workers in regard to the optimum pH for fat digestion. The results of the present study have shown that the reaction

of the medium has a great influence on the degree of digestibility of fats, usually the order of digestibility is found to be different at different pH values. Thus, with pancreatic lipase, butter-fat is less digested than other fats at pH 12.6. However, at pH 9.3, the order is completely reversed and it greatly resembles that obtained with castor-seed lipase under optimum conditions. Further the pH activity relationship of the lipolytic digestion has revealed a strikingly interesting phenomena, namely, the existence of two pH optima, one occurring at pH 9.3 and the other at pH 12.6. This novel behaviour of the lipase towards the digestion of fats has not been noticed before by previous workers, probably because in their experiments the pH limit was restricted to 10.0 and below. It is, therefore, suggested that the existence of two pH optima is perhaps due to the presence of two lipases. This explanation seems more reasonable since such types of enzymes are found to exist in animal and plant kingdoms (Giri and Datta, 1936; Giri and Sreenivasan, 1937). Further work is necessary to definitely establish the individuality of the two lipases in pancreas.

It is well known that bile is an important agency in activating pancreatic lipase (Whipple, 1922; Schmidt, 1927). From the results of the present studies, it would be seen that the striking property of the bile salt, sodium taurocholate, lies in its inability to act similarly on the digestibility of different fats by the lipase. Thus, the bile salt does not accelerate the digestion of sesame and ground-nut oils, while the digestion of ghee and coco-nut oil is very markedly augmented in its presence. Hence, the difference in the digestibility of butter-fat and coco-nut oil on the one hand and sesame and ground-nut oils on the other becomes more marked in the presence of bile salts. This is an important factor which may account for the relative superiority of ghee and coco-nut oil as compared with ground-nut and sesame oils.

The factors which are responsible for the differences observed in the digestibility of fats are not well understood. Several authors have tried to correlate digestibility with melting points, iodine values, saponification values, etc. The results of the present investigation clearly show that digestibility is greatly influenced by pH and the presence of other associated substances.

The amount of fat digested by the lipase depends also to a large extent on the surface which is exposed to the action of the enzyme. The emulsification of fats is greatly facilitated by the presence of substances which reduce the interfacial tension. In the absence of such substances the free fatty acids themselves to a certain extent aid emulsification, but the higher fatty acids are insoluble and therefore have very little influence in promoting a stable emulsion-formation. In the present investigation, it has been frequently observed that emulsion formed when butter-fat is digested with the enzyme in the absence of emulsifying agents is markedly stable for a greater length of time than those with other fats. This peculiar and unique property of butter-fat has been observed even in the case of digestion with castor-seed lipase. This would indicate the superiority of butter-fat as an easily digestible substance even in the absence of suitable emulsifying agents. With pancreatic lipase at pH 12.6, the emulsion with ghee is very unstable and this may be one of the reasons for its inferiority compared to other oils in digestibility at that pH. It is proposed to investigate quantitatively the degree of stability of the emulsion formed during the digestion of butter-fat and the other oils. This

characteristic property of the fats may be useful in devising a method for the detection of adulteration of ghee.

It is apparent from the results presented above that, on the whole, butter-fat and coco-nut oil are more easily digested than sesame and ground-nut oils (*cf.* Godbole and Sadgopal, 1935). The digestion tests have been made, as far as possible, under body conditions of temperature, fat emulsification, and H-ion concentration. While the above results are sufficient in themselves to credit such a conclusion, in view of the complexity of the problem, they cannot be applied too rigorously to the field of human nutrition. However, it is reasonable to advocate the use of coco-nut oil in place of butter-fat for edible purposes.

SUMMARY.

The digestibility of edible fats like castor-seed and pancreatic lipases has been studied under various conditions of temperature, H-ion concentration and fat emulsification. The study has led to the following important conclusions :—

1. The pH-activity relations of pancreatic and castor-seed lipases are different from one another. The variation of activity with pH depends also on the buffer used. With pancreatic lipase using ammonia-ammonium chloride buffer a sharp maximum in activity is shown at about pH 8.6, while with glycine buffer two maxima, one at pH 9.3 and the other at pH 12.6, are observed. This phenomena has been noticed with all the fats investigated. This is probably due to the presence of two lipolytic enzymes in pancreatic extract whose optimum pH are different from one another.
2. The order of relative digestibilities of the fats by pancreatic lipase at pH 9.6 is completely reversed at pH 12.6.
3. The digestibility of fats by castor-seed lipase is inhibited by bile salts, while the pancreatic digestion is enhanced in their presence.
4. Fat emulsification in the course of digestion differs markedly in the case of ghee and other oils. The relation between the stability of emulsions during digestion and the nature of the oils is discussed.
5. Sodium taurocholate shows a selective action in its acceleration effect on fats in presence of pancreatic lipase. The digestion of butter-fat and coco-nut oil is augmented, while sesame and ground-nut oils are not affected by the presence of bile salts.
6. The rate of digestion of ghee and other oils has been studied and evidence adduced to show that ghee and coco-nut oil are more rapidly digested than sesame and ground-nut oils by both castor-seed and pancreatic lipases.

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THE STABILIZATION OF VITAMIN C BY PYROPHOSPHATE.

BY

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IN the course of recent work (Giri, 1937) on the influence of vitamin C on phosphatases, it was observed that the hydrolysis of pyrophosphate by the enzyme was not influenced by the vitamin, while the glycerophosphate hydrolysis was inhibited in its presence probably due to the oxidation of the vitamin by traces of Cu present in the reaction mixture. Even in presence of minute traces of added Cu the vitamin inhibited the hydrolysis of pyrophosphate to a less extent than that of glycerophosphate. This behaviour was interpreted to mean that pyrophosphate may have a higher affinity for the metal Cu than has ascorbic acid, and prevent effective contact between the two, thereby protecting the vitamin against oxidation. This suggestion led to a further investigation of the rôle of pyrophosphate in the reaction.

It is well known that pure vitamin C (ascorbic acid) in solution is very easily oxidized under aerobic conditions, and minute traces of metals like Cu cause rapid oxidation. In tissues and biological fluids ascorbic acid is present mostly in its reduced form (Bacharach, Cook and Smith, 1934 ; Bessey and King, 1933 ; Gabbe, 1934) although copper is present in measurable quantities. Inhibitory mechanisms which protect ascorbic acid from oxidation have been found to exist in body fluids and tissues. From the work of de Caro (1934) and de Caro and Giani (1934) it is known that ascorbic acid in phosphate buffer (pH 7.5) remains unoxidized in presence of air for several hours if mixed with small amounts of liver, muscle, blood, ovary, nerve, or suprarenal gland. They concluded that glutathione was the substance which caused stabilization of ascorbic acid in tissue extracts. Mawson (1935), however, stated that protection by tissue extracts could not wholly be explained by the presence of GSH, cysteine, cystine or H_2S since dialysed tissue extracts retained their anti-oxidative properties in full. Even the extracts that had been boiled or precipitated with mercuric acetate were found to retain their anti-oxidative properties. Mawson (*loc. cit.*), therefore, suggested that 'the protective properties of tissue extracts and of

the pure compounds are probably due to their inhibition by metallic catalysts, which are present in body fluids in such a concentration that without a protective mechanism of some kind ascorbic acid could never exist in them at all'. Barron, Barron and Klemperer (1936) have recently reported the results of their experiments on the oxidation of added ascorbic acid in normal biological fluids with and without added CuCl_2 . They found that blood serum, milk, and tomato juice had the greatest protective power against added CuCl_2 , urine, saliva, and grape-fruit juice the least. Very recently Macfarlane (1936) has studied the stabilizing action of several sulphur containing compounds, and observed that the catalysis of ascorbic acid oxidation by copper is inhibited by sodium diethyldithiocarbamate, cystine, cysteine and glutathione, but not by taurine or glycine. Hopkins and Morgan (1936) have also demonstrated that glutathione completely protects ascorbic acid from oxidation by copper catalysis.

In plants, however, the existence of such protective mechanism is very rare. Damodaran and Nair (1936) found that Indian gooseberry (*Phyllanthus emblica*) contains a tannin which has an inhibitory action on oxidation of ascorbic acid solutions in air and is therefore responsible for the resistance to oxidation of the ascorbic acid in the press juice. Barron, Barron and Klemperer (*loc. cit.*) found that tomato and grape-fruit juice protected the vitamin from oxidation by copper catalysis. According to McHenry and Graham (1935) vegetable pulp has no mechanism for stabilizing ascorbic acid.

In view of the wide distribution of the vitamin and metal catalysts, the existence of protective mechanism against the oxidation of vitamin C in plant and animal tissues is of great importance. The present paper reports the results obtained in a study designed to test the possibility that pyrophosphate may protect the vitamin against oxidation. If this were the case, it would go far towards explaining the observed protective action of tissues and tissue extracts. Further, in view of the wide occurrence of pyrophosphate in plant and animal tissues (Lohmann, 1928a, b; Boyland, 1930), any evidence for the rôle of pyrophosphate in plant and animal metabolism is of considerable interest.

EXPERIMENTAL.

The method employed consists essentially in allowing pure ascorbic acid (B. D. H.) to be oxidized either alone or in presence of varying amounts of copper and trichloroacetic acid (5 per cent) and seeing whether added pyrophosphate has any protective action on the oxidation of the vitamin.

Ascorbic acid was determined by the Tillmans-Harris technique (Harris, Birch and Ray, 1933) and also by iodine titration. In order to avoid the errors arising out of any difference owing to slight variations in time and to make the end point sharper, a drop of glacial acetic acid was added to the titration flask at the time of titration, as suggested by Ahmad (1935).

The sodium pyrophosphate used in the present study was prepared by heating Sorensen's secondary sodium phosphate to a red heat in a platinum dish. The aqueous solution of the salt so prepared gave a white precipitate without any trace of yellow, with silver nitrate, and contained no orthophosphate.

Ascorbic acid solutions were prepared by dissolving the vitamin in glass distilled (thrice) water.

(i) *Protective action of pyrophosphate against the oxidation of vitamin C.*

The oxidation of ascorbic acid was followed at pH 7.2 with and without added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and the effect of pyrophosphate on the stability of the vitamin was studied. Ascorbic acid was dissolved in water contained in 50-c.c. flasks. The reaction was adjusted to pH 7.2 by addition of phosphate buffer (M/15). Copper sulphate was added in such concentrations as to bring the total amount of Cu^{++} contained in 10 c.c. of the reaction mixture to 0.032 mg. The concentration of pyrophosphate in the total volume of the reaction mixture amounted to 0.5 per cent. The reaction was allowed to proceed at $35^\circ \pm 0.1^\circ\text{C}$. in a thermostat. Aliquots were removed at various intervals and the ascorbic acid determined by titration with indophenol and iodine.

TABLE I.

The effect of pyrophosphate on the oxidation of vitamin C in presence and absence of Cu.

Amount of ascorbic acid, 5 mg.; 5 c.c. of phosphate buffer (M/15) pH 7.2; pyrophosphate, 0.5 per cent; Cu, 0.032 mg. in 10 c.c. of the mixture. Total volume of the reaction mixture, 20 c.c.; temperature, $35^\circ \pm 0.1^\circ\text{C}$.

Time in minutes.	ASCORBIC ACID, PER CENT.			
	Control.	Pyrophosphate.	Cu.	Cu + Pyrophosphate.
0	100	100	100	100
10	78	100	57	97
20	68	100	54	93
40	61	100	40	85
60	52	97	31	76
120	35	90	17	66
360	4	69	0	35

In Table I and Fig. 1 are shown the rate of oxidation of the vitamin in presence and absence of added copper and pyrophosphate. In the absence of pyrophosphate the vitamin is very easily oxidized, and in its presence the

As can be seen in Table II, pyrophosphate added to trichloroacetic acid is an excellent stabilizing agent for use in the extraction and determination of vitamin C from plant and animal materials. A somewhat unexpected result of these experiments is the inhibiting effect of Cu on the oxidation of the vitamin in trichloroacetic acid. The oxidation of ascorbic acid in aqueous solutions is generally catalysed by traces of copper, while in the present case, however, the oxidation of the vitamin in trichloroacetic acid is inhibited by Cu. An attempt to further investigate these seemingly contradictory results on the behaviour of Cu in relation to the oxidation of ascorbic acid will be made below.

(iii) *Catalytic oxidation of vitamin C in trichloroacetic acid by varying amounts of copper salt.*

The results presented in Table II show that the oxidation of the vitamin in trichloroacetic acid in presence of Cu is less than in its absence, which is contrary to the behaviour of Cu as catalyst in the oxidation of the vitamin in pure aqueous solutions (Table I). The experiments were therefore repeated by adding varying amounts of copper sulphate to ascorbic acid, trichloroacetic acid mixtures, and testing the rate of oxidation of the vitamin.

TABLE III.

Effect of varying amounts of Cu in trichloroacetic acid on the oxidation of vitamin C.

Ascorbic acid, 5 mg.; trichloroacetic acid, 5 per cent; total volume of the reaction mixture, 25 c.c.; room temperature, 26°C. to 27°C.

Concentration of Cu in 10 c.c. of reaction mixture, mg.	ASCORBIC ACID IN MG.		
	Time in hours.		
	0	5	24
0	5.0	4.0	0.95
0.025	5.0	4.42	2.33
0.050	5.0	4.45	2.33
0.125	5.0	4.34	2.18
0.250	5.0	3.96	1.91

The results presented in Table III, while confirming the previous observations, show that at low concentrations of Cu the oxidation is inhibited. This phenomenon was repeatedly observed in a number of experiments, but no explanation can at present be offered. It seems probable, however, that Cu inhibits the action of another factor present in trichloroacetic acid, which protects the vitamin from atmospheric oxidation.

(iv) *Effect of varying amounts of pyrophosphate on the catalytic oxidation of vitamin C by Cu.*

In Table IV are presented the results of experiments in which the vitamin was oxidized in acetate buffer, pH 5.0, with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ as catalyst, and in presence of varying amounts of pyrophosphate. The rate of oxidation in presence of Cu as catalyst was compared with that of identical mixtures containing varying amounts of pyrophosphate.

TABLE IV.

Effect of varying amounts of pyrophosphate on the oxidation of vitamin C with Cu as catalyst.

Ascorbic acid, 5 mg.; acetate buffer (M/5) pH 5.0, 10 c.c.; Cu, 0.032 mg. in 10 c.c. of the mixture; temperature, $35^\circ \pm 0.1^\circ\text{C}$. Total volume of the reaction mixture, 20 c.c.

Concentration of pyrophosphate, per cent.	ASCORBIC ACID IN MG. PRESENT IN THE TOTAL VOLUME OF THE REACTION MIXTURE AFTER INCUBATION AT $35^\circ \pm 0.1^\circ\text{C}$.			
	Time in minutes.			
	0	30	60	120
0	5	3.3	2.6	1.6
0.05	5	3.6	3.1	2.5
0.125	5	3.6	3.1	2.5
0.250	5	3.6	3.2	2.6
0.500	5	3.9	3.4	2.8
1.250	5	4.8	4.5	4.3

It can be seen from Table IV that with the increase in the concentration of pyrophosphate its inhibiting power also increases. Thus the ratio between the pyrophosphate and Cu concentrations determines the degree of protection by pyrophosphate. It is, therefore, evident that pyrophosphate combines with Cu to form a complex, thereby preventing it from catalysing the oxidation of the vitamin. It is probable that the complex compound formed by pyrophosphate with Cu may be an un-ionized copper complex, and hence the inhibition of oxidation of the vitamin by Cu may be due to the fact that the un-ionized copper does not catalyse the oxidation (Barron, Barron and Klemperer, *loc. cit.*).

(v) Influence of added pyrophosphate on the stability of vitamin C contained in lemon juice.

Lemons are frequently used in human diet as a source of vitamin C that any information regarding the stability of the vitamin present in the juice obtained from them is of interest. Experiments were therefore conducted with lemon juice with a view to finding out whether pyrophosphate when added to fresh lemon juice exerts any stabilizing action on the vitamin. Fresh lemons were used for these experiments. The juice was removed by squeezing and the extracted juice was strained through cloth. Immediately pyrophosphate solution was added to one lot and an equal volume of water was added to the other and at stated intervals the samples were tested for its vitamin content by titration with indophenol. The results of these experiments are listed in Table V:—

TABLE V.

Time the solution was allowed to stand at room temperature (25°C. to 26°C.).	VOLUME OF THE SOLUTION REQUIRED TO REDUCE 1 C.C. OF 0.05 PER CENT INDICATOR.		
	10 c.c. of lemon juice + 10 c.c. water.	10 c.c. of lemon juice + 6 c.c. of 5 per cent pyrophosphate + 4 c.c. water.	10 c.c. of lemon juice + 10 c.c. of 5 per cent pyrophosphate per cent.
0 hour	1.13	1.11	1.11
3 hours	1.21	1.11	1.11
8 „	1.54	1.17	1.14
24 „	3.08	1.81	1.66

These results show that addition of pyrophosphate to lemon juice inhibits the destruction of vitamin C.

DISCUSSION.

The results presented in this paper are of interest in view of the possible explanation which they provide for the protective action of animal tissues and tissue extracts against the oxidation of vitamin C. Svirbely (1936) has recently drawn attention to the biological importance of the relation of ascorbic-acid content of the organs to the amount of copper sulphate fed to the rats. His experiments have shown that the feeding of copper sulphate does not prevent the synthesis of vitamin C in the rat. He suggests therefore that the body has a protective mechanism which prevents the catalytic oxidation of vitamin C with copper sulphate. It is generally assumed that glutathione present in tissues is responsible for the protective action of tissue extracts (de Caro and Giani, *loc. cit.*). According to Mawson (*loc. cit.*), however, the protective action of tissues and tissue extracts is not

quantitatively accounted for on the basis of their content of sulphydryl compounds. Therefore the existence of other hitherto unknown protective mechanisms in animal tissues is evident from the results of the foregoing workers. Now it is known that pyrophosphate occurs widely in plants, animals and yeast (Lohmann, 1928*a*, *b*). The results obtained in the present study show clearly that pyrophosphate protects the oxidation of ascorbic acid by copper catalysis. Thus, the existence of another protective mechanism in addition to that of glutathione, against the oxidation of vitamin C in plant and animal tissues, is established.

Further the results obtained in the present study are of sufficient interest in their application to the determination of ascorbic acid in plant and animal products, and in the preparation of stable aqueous solutions of the vitamin. The method generally adopted for the determination of vitamin-C content of plant materials is to extract the vitamin by grinding the material with trichloroacetic acid, centrifuging the extract and titrating it with sodium 2 : 6 dichlorophenolindophenol. The plant tissues, the reagent, and the water used may contain copper and other metallic impurities which catalyse the oxidation of the vitamin (Kellie and Zilva, 1935), and it is impracticable to remove every trace of copper from all reagents and water used in the extraction and estimation of the vitamin. In order to reduce errors in the method for the determination of the vitamin, the time consumed in conducting the titrations is an important factor, since the vitamin is easily oxidized in presence of air. Cheftel and Pigeand (1936) have, therefore, suggested the carrying out of titrations of ascorbic acid with indophenol at 0°C., since oxidation is much slower at this temperature. The addition of certain reagents to the extractant, which inhibit the catalytic oxidation of the vitamin by copper, also suggests itself. The reagents which are known to inhibit the catalytic activity of copper in the oxidation of ascorbic acid are potassium cyanide, H_2S , cystine hydrochloride, glutathione, and metaphosphoric acid. KCN and H_2S are effective only in weak acid solutions. Further H_2S , being in itself a reducing agent, must be completely removed before titrating with the indophenol. Tauber and Kleiner (1935) have suggested the use of cystine hydrochloride as a stabilizing agent, which, however, possesses few advantages in view of the fact that its inhibiting effect has been found to be largely due to the lowered pH accompanying the addition of excess HCl necessary to dissolve the cystine rather than by any specific action of the reagent (Mack and Kertesz, 1936). Glutathione is, of course, an efficient stabilizing agent, but its prohibitive cost prevents its use as a reagent for the determination of the vitamin. The only other efficient stabilizing agent which has been successfully employed by many workers in vitamin-C studies is metaphosphoric acid. Fujita and Iwatake (1935) were the first to suggest that metaphosphoric acid exerts a stabilizing action on the vitamin and that it can be used for its extraction. Subsequent workers have reported better results by using metaphosphoric acid either alone (Levy, 1936; Hinsberg, 1937) or in combination with trichloroacetic acid (Musulin and King, 1936) for stabilizing and extracting the vitamin from plant and animal tissues. Musulin and King (*loc. cit.*) recommend the addition of 2 per cent metaphosphoric acid to trichloroacetic acid for extraction of the vitamin. The metaphosphoric acid, whilst preventing oxidation due to enzyme activity and traces of copper, does not interfere with the estimation of the vitamin by indophenol titration. Very recently Lyman, Schultze and King (1937) have investigated the possibility of using neutralized metaphosphoric acid in the buffers with a view

ON THE ESTIMATION OF ATEBRIN IN TISSUES.

BY

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CHOPRA and ROY (1935) described a method for the estimation of atebtrin in the blood. Chopra, Ganguli and Roy (1936) also worked out its concentration in the circulating blood at different intervals of time after the administration of the drug, in cases of monkey malaria. Attempts were then made to determine how atebtrin is distributed in the different tissues and whether any particular organ had a greater pre-selection for the drug than the others. The extraction of small quantities of alkaloids and other organic substances from tissues presents some difficulties and the procedure in each case must necessarily vary according to the nature of the substance. Two kinds of procedure are usually employed. The substance in question may either be extracted from the minced tissues by means of a solvent with simultaneous coagulation of the proteins, for instance, hot acidified ammonium sulphate, or the protein materials may be dissolved by some agents which have no action on the substance to be determined and then the substance extracted from it by means of a suitable solvent. The first of these procedures is time-consuming and also admits of considerable errors. It was applied by Ramsden *et al.* (1918) for the estimation of quinine from tissues, but this method is inapplicable in the case of atebtrin, because atebtrin itself is insoluble in acidified ammonium sulphate. The treatment of the minced tissues by means of ether also results in incomplete extraction of the substance.

We had therefore to take recourse to the other alternative. The more drastic methods of dissolving out the tissue materials, by means of strong alkali for instance, we were reluctant to employ as we thought that such a procedure might partially or completely destroy atebtrin itself. We therefore tried to get

the protein material into solution by treatment with one of the proteolytic enzymes. Papain appeared to us to be more suitable for the purpose as it was found to act almost equally well in acid, alkaline, or neutral reactions, so far as the digestion of meat is concerned. The tissue was therefore digested by means of papain and the atebrin was estimated by Tropp and Weise's (1933) method applicable to urine.

Procedure.—The weighed tissue is minced, taken in an Erlenmeyer's flask of suitable capacity and a small amount of papain (powder), 0.2 g. to 1 g. depending upon the weight of the tissue, is sprinkled over it and well mixed. It is then kept in this condition for 10 to 15 minutes with frequent mixing of the materials. Approximately N-10 HCl is then added (5 c.c. of the acid for 1 gramme of the tissue) and the contents of the flask were mixed and put in a water-bath maintained at 60°C. for 1 hour or more if the tissues are not completely digested and stirred from time to time. Muscles are more or less completely digested by this procedure but some of the organs such as liver, lung, etc., which contain considerable amount of fat, appeared to be comparatively unaffected. What appeared to be undigested residue, however, could be easily reduced to a fine pulp by means of a glass-rod, which formed a fine sediment at the bottom which can be easily washed to remove atebrin. It is then centrifuged, the clear supernatant fluid is transferred to a separating funnel and the residue washed 2 or 3 times with a suitable amount of N-10 HCl. After centrifuging each time the clear fluid is transferred to the funnel. It is then alkalized with 60 per cent KOH and the mixture thoroughly shaken with 20 c.c. to 30 c.c. of ether (depending upon the volume of the extract). It sometimes happens that there is not a clear separation in two layers and a gelatinous intermediate layer is formed which demixed only very slowly. In such a case the clear aqueous solution is drained and 4 c.c. to 5 c.c. of 96 per cent alcohol are added into the funnel. The whole is again thoroughly mixed and the mixture is now clearly separated in two distinct layers. Now the mixture is again separated and the ether collected apart and the remainder of the solution is again shaken with 20 c.c. to 30 c.c. of ether. The extraction of atebrin is usually complete at this stage. The united ether extracts are twice washed with 20 c.c. to 30 c.c. of water. Any coloration of the wash-water, if there be any, has nothing to do with atebrin. The ether extract is then shaken with two successive 10 c.c.-lots of N-10 HCl, whereby the ether becomes entirely colourless. The acid extract is made up to a convenient volume with N-10 HCl, depending on the atebrin content, and compared in a colorimeter against a standard solution of atebrin. A 0.01 per cent solution of atebrin in N-10 HCl serves as a stock standard and it may be conveniently diluted to match the colour of the unknown.

In case of tissues containing very small quantities of atebrin, procedure similar to that used in the case of blood is to be employed, viz., the final hydrochloric acid extract or an aliquot portion of it is alkalized and taken up in equal amounts (0.5 c.c.) of amyl alcohol and compared with a set of standards containing varying amounts of atebrin similarly treated.

This method appears to us to be quite simple and fairly accurate and does not admit of an error of more than 15 to 20 per cent.

When this work was almost complete Prof. Schulmann was kind enough to send us a photo copy of Dr. Hecht's paper on 'the distribution of atebrin in the

organism' which has been subsequently published (1936). In this paper Dr. Hecht has described a method for the estimation of atebtrin in small quantities of blood (1 c.c.) and different tissues (1 gramme) and has made an elaborate study of the distribution of the drug in various organs and tissues. The principle underlying the method consists of the dissolution of blood or tissue by the addition of equal quantities of 60 per cent caustic potash and heating in a boiling water-bath for 5 minutes. Then distilled water equal in amount to the KOH solution used is added to reduce it to a homogeneous fluid mass which is then extracted with a mixture containing 8 parts of benzene and 2 parts of amyl alcohol (2 c.c. per 1 gramme of the organ). After centrifugation 75 per cent of the volume of the solvent was taken off and shaken with a measured amount (1.5 c.c.) of N-1 HCl. After it has settled down (eventually centrifuged), 1 c.c. of HCl solution is removed from below the solvent and the colour comparison made according to the technique employed by Chopra and Roy (1936), viz., the acid solution is alkalinized and shaken with equal small quantities of amyl alcohol and the colour compared with a set of standard atebtrin solution similarly treated.

It appears that the difference between Hecht's technique and that of Chopra and Roy consists only in the process of extraction of atebtrin from blood or other tissues. In our first communication on this subject (1935), filter-paper strips were soaked with a measured amount of blood which were then heated at a temperature of 50°C. for an hour and extracted with ether in an extraction apparatus. Later on (1936) we introduced certain modifications (the paper strips containing the blood were dried by putting inside a desiccator overnight) which were found to be advantageous. Experiments with known amounts of atebtrin by this technique gave fairly satisfactory results. The use of a strong alkali solution for the partial dissolution of the proteins did not appear to us to be sound in principle as atebtrin itself might be partially destroyed or altered in some manner by this treatment. Hecht has remarked that 'about 10 per cent of the atebtrin is destroyed by this procedure'. We, however, applied Hecht's technique in the case of blood and found it quite satisfactory and it has also certain advantages over the procedure employed by Chopra and Roy in that the former is simpler, does not necessitate the use of a special extraction apparatus and the determinations could be carried out within a much shorter time. The recovery of the added atebtrin is also quite good.

When, however, we try to apply this method to organs and other tissues, we find that it is beset with certain difficulties. Hecht has evidently meant his technique to be applied only to small quantities of the tissues. Now it is a well-known fact that the distribution of a drug is not uniform throughout the organ and it is probable that different parts may have different concentrations of atebtrin. The values of atebtrin obtained from 1 gramme of an organ taken at random from the whole, which in the case of a cat's liver, for instance, may weigh 50 grammes to 60 grammes or even more, do not give a fair estimate of the total atebtrin content of the organ as a whole and the chances of error will necessarily be great.

Moreover, the essential feature of both these processes is to determine atebtrin content from the intensity of yellow colour. It is, therefore, essential to eliminate all other kinds of colouring matter excepting that due to atebtrin. We found that treatment with dilute HCl serves to extract only the atebtrin and other colouring matters are excluded. But a preliminary treatment of the organs and tissues with

hot 60 per cent caustic potash might partially destroy atebrin and also might change the pigments present in certain organs such as liver, gall bladder, lung, etc., in such a manner as to render them soluble in dilute HCl. Hecht experienced such difficulties in the case of cat's liver and gall bladder, and we on repetition of Hecht's technique found that the final amyl-alcohol layers were distinctly coloured in the case of cat's liver, gall bladder, lung, and kidney. This will introduce serious errors especially when a small quantity of the organ is dealt with.

Therefore we prefer to digest the whole organ with papain under the conditions mentioned above. In case of smaller organs weighing about 1 gramme or less (excepting, of course, liver, gall bladder, lung, and kidney) Hecht's technique is quite suitable and yields quite satisfactory results.

CONCLUSION.

A simple and fairly accurate method for the estimation of atebrin in the tissues is described.

We are thankful to Mr. Bolai Ch. Das for his valuable assistance in this connection.

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A PRELIMINARY REPORT ON THE EFFECT OF ANTI-MALARIAL DRUGS UPON THE INFECTIVITY OF THE PATIENTS TO THE MOSQUITOES.

BY

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AMONG other factors in connection with the transmission of malaria by mosquitoes, the effect of anti-malarial drugs on the viability of the gametocytes of human plasmodia and on their development in the mosquito has been studied by a number of workers and some valuable information has been obtained. The Malaria Transmission Inquiry while working out the atmospheric temperature and humidity factors in the transmission of malaria made some observations on this subject and in this paper we propose to give briefly our results.

The patients were all admitted into the Carmichael Hospital for Tropical Diseases under the senior author (R. N. C.) who was testing the effects of various anti-malarial remedies on different strains of plasmodia occurring in India. The mosquitoes used in our experiments were all laboratory-bred *Anopheles stephensi*, larvæ of which were collected from Calcutta and its environs. The clean females were fed on gametocyte-carriers (*P. vivax*, *P. malariae*, and *P. falciparum*) after treatment with various doses of different anti-malarial drugs. As a control in most cases mosquitoes were fed on the same patients before treatment. Factors such as atmospheric temperature and humidity, and numerical strength of the gametocytes have also been studied which will be discussed in another communication. Here we shall deal with only one factor, namely, effect of previous treatment.

Before proceeding to state our own findings it may be of some interest to mention briefly some of the previous works on the subject.

Bruce Mayne (1920) observed that sporogony cycle would develop after administration of 450 grains of quinine during treatment; such mosquitoes were capable of infecting healthy persons. Simanin (1928) carried out experiments with 14 malaria patients who had been taking quinine for varying lengths of time. He found that development of the parasite will occur in the mosquitoes fed on patients with B. T. and M. T. infections taking quinine in therapeutic doses. Green (1929) found that quinine does not touch the crescents. As distinct from reducing the number of gametocytes in the blood, it is somewhat doubtful whether, in addition, quinine affects the viability of the gametes of *P. vivax* and *P. malariae*. The same author (1934) carried out experiments in which anopheles were fed on patients infected with *P. vivax* and *P. falciparum* who had undergone treatment with various drugs other than plasmochin. The gametocytes continued their development in the mosquito unchecked, and it was concluded that there was no drug which affects both the crescents and ring forms of *P. falciparum*.

Strickland and Roy (1932a) noted that atabrin administered to a patient suffering from malaria completely prevented the development in the mosquito of any gametocyte from that host, and that the parasite resumed its developmental powers three days after the discontinuance of the treatment. In the same year the above authors (1932b) rectified their statement by saying '..... We have recently tried without success to repeat our experience regarding the influence of atabrin on the development of malarial plasmodia in the mosquito, reported in the April number (of the *Ind. Med. Gaz.*) of this year. Of 135 mosquitoes fed on a patient at varying times, some, soon after the administration of atabrin, 116 developed sporozoites. Green in the Federal Malay States and, we are permitted to say also, Basu in Knowles' laboratory in the Calcutta School of Tropical Medicine have obtained similar results'.

Barber and Komp (1927) showed that the gametocytes of M. T. and B. T. would not develop in the mosquitoes after two days' continued treatment with plasmochin and quinine, and pointed out the importance of this finding in the possible control of malaria by treatment. In the authors' own language 'should it prove that small doses of plasmochin may so cripple gametocytes or so far interfere with their normal development as the case may be, that they are rendered incapable of forming healthy oöcysts, the usefulness of plasmochin, or plasmochin combined with quinine, could be greatly extended'.

The same workers (1928) continued their observations and found plasmochin in small doses, in one case in a single dose of $\frac{1}{2}$ cg., to have a definite effect on the viability of crescents as measured by mosquito-infection tests. It is possible that the general use in a population of such small doses of plasmochin would be safe and effective in reducing the transmission of malaria.

Manson-Bahr (1928) showed that a single dose of 0.03 g. of plasmochin, though not sufficient to destroy the crescents, was enough to prevent their exflagellation on a glass-slide. Whitmore (1929) stated that a single dose of plasmoquine of 0.300 mg. per kilo of body-weight is sufficient to render non-infectious, for

A. albimanus, all crescents in the blood at the time the single dose is taken. His findings show that a dose of 0.200 mg. per kilo of body-weight is not sufficient to render the crescents non-infectious for *A. albimanus*. In his later experiments he ascertained 0.325 mg. as the single effective dose. Amies (1930) stated that 0.04 g. of plasmochin given in two doses of 0.02 g., sixteen hours apart, renders crescent carriers non-infective for at least three days. Sur, Sarkar and Banerjee (1932) found plasmochin even in small doses of 0.02 g. per day per adult for three days without the aid of quinine can prevent development of malaria parasites in transmitting species of anopheline mosquitoes.

Green (1934) observed that atebrin destroys the gametocytes of the benign tertian and the quartan parasite at about the same rate as does quinine or plasmoquine. It has no particular advantage as a 'gametocide' in benign tertian or quartan malaria.

With regard to crescents, there seems to be a difference between the action of either atebrin or quinine on these subtertian gametocytes. Atebrin does not prevent crescents being formed and does not cause crescents to become non-viable. A crescent-carrying patient will still infect anopheline mosquitoes during or after seven days' continuous treatment with atebrin.

The Table shows the result of our own observations. The infectivity of the gametocytes of the three species of Indian strains of malaria in the mosquitoes after treatment with various anti-malarial drugs has been dealt with separately.

In experiments with cases of *P. vivax* infection who had a fractional treatment with quinine sulphate, plasmoquine, and malarcan (*vide* Table) there was no salivary-gland infection in the mosquito; only in two experiments out of 58 mosquitoes, five developed malarial oöcysts from cases who had had previous treatment with 0.01 g. and 0.02 g. of plasmochin respectively. Clean *A. stephensi* mosquitoes, 354 in number, were used in these experiments, of which 160 survived and were examined, and only five showed gut infection. Detailed results are shown in the Table.

In experiments with cases of *P. malariae* infection who had similar treatment with atebrin, malarcan, and tebetren (as shown in the Table), there was no salivary-gland infection in any of the mosquitoes; only eight mosquitoes showed gut infection. The positive result obtained was with the tebetren case. Altogether 402 clean female *A. stephensi* mosquitoes were used in these experiments, of which 263 survived and were examined, and eight showed gut infection. Detailed results are shown in the Table.

Experiments with cases of *P. falciparum* infection who had previous treatment with different doses of cinchona febrifuge, quinine sulphate, atebrin, plasmochin, gametochin (Union Drugs), and tebetren gave interesting results. All the drugs (except plasmochin) failed to devitalize the crescents which developed up to the sporozoite stage in the mosquitoes in high percentages, but in the case of plasmochin 0.02 g. was sufficient to stop the development in the mosquito.

In all as many as 3,909 female *A. stephensi* were used for these series of experiments, of which 2,183 survived, with a result of 1,037 gut infections and 314 salivary-gland infections. For details see Table.

TABLE.
Effect of previous treatment.

Drug.	Dosage.	Experiments.	<i>A. stephensi</i> , fed.	<i>A. stephensi</i> , survived.	Gut +	Glands +	Interval in days.
<i>Benign tertian.</i>							
Quinine sulphate	1	1	118	26	0	0	7
Plasmoquine	1	1	90	52	3 = 6 per cent	0	15
"	2	1	14	6	2 = 23 "	0	14
"	3	1	58	30	0	0	14
"	4	1	27	10	0	0	13
Malarcan	28	1	47	36	0	0	7
<i>Quartan.</i>							
Atebrin	3	2	92	43	0	0	12 to 13
"	6	1	32	27	0	0	11
"	9	1	12	2	0	0	10
"	12	1	70	61	0	0	19
Malarcan	3	1	30	21	0	0	14
Tebetren	15	1	166	109	8 = 7 per cent	0	12

Malignant tertian.

Cinchona tebringe ..	1	1	40	24	11 = 46 per cent	0.	14
" "	2	1	31	19	11 = 58 "	0	13
" "	3	6	283	112	64 = 57 "	36 = 32 per cent	12 to 22
" "	9	1	25	23	14 = 61 "	8 = 35 "	9 to 16
" "	10	1	24	12	4 = 33 "	11 = 92 "	11
Quinine mixture	2	1	70	28	0	0	18
" "	4	1	40	21	0	0	18
" "	10	2	66	40	10 = 25 per cent	23 = 57 per cent	8 to 14
Atebrin	3	4	314	172	50 = 29 "	24 = 14 "	9 to 13
" "	6	5	237	129	39 = 30 "	22 = 17 "	9 to 14
" "	9	5	386	196	13 = 7 "	9 = 5 "	10 to 14
" "	12	10	454	295	116 = 39 "	62 = 21 "	7 to 17
" "	15	9	1,099	740	678 = 92 "	97 = 13 "	8 to 24
Plasmoquine	1	1	34	27	0	0	12
" "	3	1	26	16	0	0	13
Atebrin and plasmoquine.	12 to 15 2 to 4	4	113	73	2 = 3 per cent	4 = 5 per cent	9 to 23
Gametoquin	2	1	120	69	2 = 3 "	0	14
" "	4	1	120	27	2 = 7 "	0	15
" "	6	6	300	136	6 = 4 "	6 = 4 per cent	7 to 21
Teberen	9	2	28	11	7 = 63 "	6 = 54 "	14 to 21
" "	18	4	99	11	8 = 73 "	6 = 55 "	5 to 10

DISCUSSION.

Clemesha and Moore, Barber and his colleagues, Kligler, deMello, Kingsbury, Amies, Sur, and others in different parts of the world have tried to control malaria transmission in the field by adopting the principle of devitalizing the gametocytes in the human host. Some combined it with anti-larval measures also; but most of them, except in some very controlled and isolated areas, met with failure sooner or later. Schulemann (1932) expressed the opinion that anti-mosquito measures will still be necessary, as it will rarely be possible to treat every carrier in a district.

SUMMARY.

1. Laboratory-bred *Anopheles stephensi*, 4,665 in number, were fed on gametocyte-carriers (*P. vivax*, *P. malariae*, and *P. falciparum*), admitted in the Carmichael Hospital for Tropical Diseases. Experiments were carried out with a view to investigate the effect of varying doses of different anti-malarial drugs upon the infectivity of the patient to the mosquito.

2. The drugs used in these experiments were cinchona febrifuge, quinine sulphate, malarcan, tebetren, atebrin, plasmochin, and gametochin.

3. The effects of atmospheric temperature, humidity, and numerical strength of gametocyte factors are not dealt with in this paper.

4. Atebrin, quinine sulphate, cinchona febrifuge, tebetren, and gametochin failed to prevent the development of crescents in *A. stephensi*. In some cases, very heavy percentages of gut and gland infections were observed even after the usual course of treatment with these drugs. Plasmoquine in such small doses as 0.02 g. prevented further development of the crescents in *A. stephensi*.

5. Even 15 doses of tebetren failed to stop the development of gametocytes of *P. malariae* in *A. stephensi*, but 3 doses of atebrin and malarcan could stop it.

6. Plasmoquine in doses of 0.02 g. could not prevent the development of gametocytes of *P. vivax* in *A. stephensi* up to the oöcyst stage. With a small dose of quinine sulphate it was stopped.

7. This work is of a preliminary nature and is being further pursued.

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ACTION OF p-AMINO BENZENE SULPHONAMIDE AGAINST STREPTOCOCCAL INFECTIONS IN MICE.

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SINCE the observation of Tre'fouel, Nitti and Bovet (1935) that the death of animals infected with streptococci is delayed several days by ingestion or injection of a simple compound like p-amino benzene sulphonamide, a good deal of work (*cf.* Goissedet *et al.*, 1935; Colebrook and Kenny, 1936; Buttle *et al.*, 1936) has been done on this protective action of the drug and it may now be said that p-amino benzene sulphonamide possesses a bacteriostatic and bactericidal action at least against small numbers of certain hæmolytic streptococci. It would be of considerable interest to find out whether the same protection can be obtained equally well against infections with streptococci of different serological types.

EXPERIMENTAL.

p-acetamino benzene sulphonyl chloride, prepared according to Marvel *et al.* (1925), was treated with ammonia and the resulting product was hydrolysed by dilute hydrochloric acid to afford p-amino benzene sulphonamide. The substance crystallizes from boiling water in prismatic plates, m.p. 266.6°C. (corrected). It was tested on white mice infected with streptococci obtained from a case of erysipelas (local) as well as with strepto-pyogenes (hæmolytic). For comparison, the effects of the strains from streptococci isolated from a local case of puerperal sepsis and of those from scarlet fever (foreign) were also observed. The different strains have been repeatedly passed through mice before being used in the present investigations. A minimal lethal dose of each strain was, of course, first ascertained by injecting different doses of the living cultures on different groups of animals. The actual injecting dose was usually four to five times the minimal

lethal dose and in all the experiments 7.5 mg. of the compound in a sterile aqueous solution were subcutaneously administered an hour before injecting the mice with the living cultures of the respective streptococci. An unprotected set of animals was kept for control. Tables I to IV show the results of the therapeutic experiments :—

TABLE I.

Strain : strepto-hæmolytic (erysipelas). Average weight of mice : 10 g. to 12 g.

Number of organisms : 2,000^a approx. (18 hours' broth culture) per c.c.

Infection dose : 0.75 c.c.

Number of mice.		24 hours.	48 hours.	72 hours.	96 hours.	Autopsy and blood culture report of the dead mice.
4	Injected subcutaneously with the drug before being infected intraperitoneally with the streptococci.	All living	All living	All living	All living	..
4	Infected intraperitoneally with the streptococci.	3 dead, 1 surviving.	1 surviving	1 surviving	1 surviving	Strepto-hæmolytic

TABLE II.

Strain : strepto-pyogenes (hæmolytic). Average weight of mice : 10 g. to 12 g.

Number of organisms : 3,000^a approx. (18 hours' broth culture) per c.c.

Infection dose : 0.5 c.c.

Number of mice.		24 hours.	48 hours.	72 hours.	96 hours.	Autopsy and blood culture report of the dead mice.
4	Injected subcutaneously with the drug before being infected intraperitoneally with the strain.	3 dead, 1 living.	1 living	1 living	1 living	Strepto-hæmolytic
4	Infected with the strain	All dead	"

TABLE III.

Strain : strepto-haemolytic (puerperal septicæmia). Average weight of mice : 10 g. to 12 g.
 Number of organisms : 3,000^a approx. (18 hours' broth culture) per c.c.
 Infection dose : 0.5 c.c.

Number of mice.		24 hours.	48 hours.	72 hours.	96 hours.	Autopsy and blood culture report of the dead mice.
4	Injected subcutaneously with the drug before being infected intraperitoneally with the streptococci.	1 dead	3 living	3 living	3 living	Strepto-haemolytic
4	Infected with the streptococci.	All dead

TABLE IV.

Strain : strepto-haemolytic (scarlatina). Average weight of mice : 10 g. to 12 g.
 Number of organisms : 3,000^a approx. (18 hours' broth culture) per c.c.
 Infection dose : 0.5 c.c.

Number of mice.		24 hours.	48 hours.	72 hours.	96 hours.	Autopsy and blood culture report of the dead mice.
4	Injected subcutaneously with the drug before being infected intraperitoneally with the streptococci.	All alive	All alive	All alive	All alive	..
4	Infected intraperitoneally with the streptococci.	1 dead, 3 living.	1 dead, 2 living.	2 living	2 living	Strepto-haemolytic

A striking phenomenon in the nature of development of nervous symptoms was noticed while injecting a group of animals with a solution of the sulphonamide that was left aside in an ampoule for a few weeks on the working table. The solution

had not undergone any apparent change excepting a slight alteration in colour, nor the substance present in it had altered to any extent, as the melting point of the substance obtained on evaporating the solution of the ampoule was found to remain unaltered when admixed with an equal portion of an authentic sample of *p*-amino benzene sulphonamide. The symptoms varied from slight ataxic gait, tremors to severe convulsions leading to death in certain cases. Of course, this simple compound is not absolutely non-toxic (*cf.* Foulis and Barr, 1937) and is often found to produce sulphæmoglobinæmia (Discombe, 1937). Investigations for finding out the ætiology of the above unusual symptoms are, however, in progress.

An attempt was next made to study the action of the drug *in vitro* upon broth cultures. The following two tables give the results of these experiments:—

TABLE V.

10 c.c. of nutrient broth.	24 hours.	48 hours.	72 hours.	96 hours.
Strepto-hæmolytic (scarlet) .. {	Heavy (1,000 ^a)	Heavy (5,500 ^a)	Heavy (7,500 ^a)	Heavy (7,500 ^a)
„ + 7.5 mg. amide .. {	Slight (250 ^a)	Heavy (5,000 ^a)	} „	} „
Strepto-hæmolytic (puerperal septicæmia) {	Heavy (2,000 ^a)	Heavy (7,000 ^a)	Heavy (7,500 ^a)	Heavy (7,500 ^a)
„ + 7.5 mg. amide .. {	Slight (100 ^a)	Slight (250 ^a)	Slight (250 ^a)	Slight (250 ^a)
Strepto-hæmolytic (erysipelas) .. {	Heavy (1,500 ^a)	Heavy (3,000 ^a)	Heavy (5,500 ^a)	Heavy (6,000 ^a)
„ + 7.5 mg. amide .. {	„	„	Heavy (5,000 ^a)	} „
Strepto-hæmolytic (pyogenes) .. {	Heavy (2,000 ^a)	Heavy (7,500 ^a)	Heavy (7,500 ^a)	Heavy (7,500 ^a)
„ + 7.5 mg. amide .. {	„	Heavy (5,000 ^a)	} „	} „

TABLE VI.

10 c.c. of nutrient broth.	24 hours.	48 hours.	72 hours.	96 hours.
Strepto-hæmolytic (scarlet) ..	Heavy (2,500°)	Heavy (7,500°)	Heavy (7,500°)	Heavy (10,000°)
„ + 10 mg. amide ..	Fair (1,000°)	Fair (2,000°)	Heavy (5,000°)	Heavy (7,500°)
Strepto-hæmolytic (puerperal septicæmia) {	Heavy (5,000°)	Heavy (5,000°)	Heavy (7,000°)	Heavy (7,500°)
„ + 10 mg. amide ..	Nil	Nil	Nil	Nil
Strepto-hæmolytic (erysipelas) ..	Heavy (5,000°)	Heavy (5,500°)	Heavy (7,500°)	Heavy (10,000°)
„ + 10 mg. amide ..	Nil	Slight (100°)	Slight (250°)	Slight (250°)
Strepto-hæmolytic (pyogenes) ..	Fair (1,500°)	Heavy (5,500°)	Heavy (5,500°)	Heavy (7,500°)
„ + 10 mg. amide ..	Slight (250°)	Fair (3,000°)	Heavy (5,000°)	„

DISCUSSION.

From all the above tables it is evident that p-amino benzene sulphonamide has definitely protected mice against infections produced by some of the different strains of streptococci. The best protective action is found in the group infected with strepto-hæmolytic (puerperal septicæmia) strain (Table III). Here all of the unprotected and one of the protected died showing a 75 per cent survival amongst the treated animals. The next best result is found in the group infected with strepto-hæmolytic (erysipelas) strain (Table I) where three of the unprotected died, whereas all the protected animals survived. From Table IV, it is clear that the protective action against strepto-hæmolytic (scarlatina) is not so marked as 50 per cent of the unprotected animals also survived. Lastly, in the group infected with strepto-pyogenes (Table II) 75 per cent death even amongst the protected animals tends to indicate an inefficacy of the drug. It will be noticed that there is a fair agreement between these observations and those obtained in test-tube experiments (*vide* Tables V and VI). But it may again be pointed out that this drug, being capable of producing sulphæmoglobinæmia, should be cautiously

prescribed even in puerperal septicæmia cases, especially in India where a noticeable anæmia is often found to be present in a considerable percentage of women. For this, a study with other types of sulphonamide compounds has been contemplated, and a work in this direction is already in progress.

CONCLUSION.

It would be unwise to make an attempt to draw up a list of conclusions from the examination of so small a number of animals, but it is permissible to say that we obviously have in *p*-amino benzene sulphonamide a powerful therapeutic agent at least for the treatment of infections caused by strepto-hæmolytic puerperal septicæmia and erysipelas. Of course, it must be said that no explanation could be put forward for this selective protection against these two particular strains of streptococci.

Lastly, the authors wish to express their thanks to Mr. A. K. Choudhury, M.Sc., of this Laboratory for much help in the course of this work.

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ADSORPTION OF ANTIGENS BY ANTIBODIES OR VICE VERSA.

Part IV.

THE EFFECT OF DIFFERENT FACTORS ON THE FLOCCULATION OF CONCENTRATED ANTITOXINS OF TETANUS AND DIPHTHERIA IN THE PRESENCE OF THEIR RESPECTIVE TOXINS.

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It has been stated (Marrack, 1934) that diphtheria antitoxin concentrated by precipitation with sodium sulphate does not flocculate when mixed with an equivalent quantity of the toxin. As this flocculation reaction furnishes an easy means of determining the potency of an antitoxin *in vitro*, it will be useful if a method could be found by which diphtheria antitoxin precipitated by sodium sulphate can also be made to flocculate. In the case under consideration there seem to be two possible factors which acting either singly or jointly might inhibit flocculation, viz., (i) the adsorption by the antibody particles of some impurities which increase their hydrophil character and (ii) the accumulation in the system of a large amount of non-specific proteins in the course of precipitation. If that be so their elimination is essential for bringing about the flocculation of the antigen-antibody mixture. Loeb (1918) has shown that the adsorbing power of a protein is minimum at its iso-electric point. This suggests a probable method of removing any impurities which might have been adsorbed by the antibody in the course of precipitation, by adjusting the pH of the solution to the value obtaining at the iso-electric point.

Furthermore, if it be assumed that the adsorbed impurities are primarily responsible for inhibiting flocculation it follows that, after they have been removed by elution, a balanced mixture of the antitoxin and the toxin should flocculate, although the reaction under such conditions may not be very strong. The results obtained seem to justify this conclusion.

EXPERIMENT.

Flocculation of diphtheria antitoxin concentrated by precipitation with sodium sulphate.

One hundred and fifty c.c. of fresh diphtheria antiserum were diluted to 300 c.c. with water and to this was added 300 c.c. of 26 per cent solution of sodium sulphate. The mixture was kept in an incubator at 37°C. until the precipitate settled down. The supernatant solution was filtered and to the filtrate enough sodium sulphate (anhydrous) was added to bring its concentration to 22.5 per cent. The precipitate obtained at this stage contained practically the whole of the antibody and was separated from the supernatant liquid by filtration. The precipitate was mixed with a small quantity of water and freed from sodium sulphate by dialysis against water in a cellophane bag. The solution of antitoxin was diluted ten times, its pH adjusted to 5.4, and left in a refrigerator at 4°C. for three days. After this period the clear supernatant solution was withdrawn and was divided into two portions. To one portion, sodium chloride and to the other sodium citrate were added until their concentrations in the solutions were 0.9 per cent and 3.4 per cent respectively. Flocculation tests were then carried out on each of these samples and were found to be successful in each case. In all three lots of antitoxin concentrated by the method described above were tried. The fresh antisera were obtained from the test-bleeding of horses under immunization. The results are recorded in Table I. It will be noticed from these data that, in each case, the potency determined by the flocculation test agrees well with that determined by animal experiments.

TABLE I.

Sample number of concentrated antitoxin.	Units per c.c. determined by flocculation test.	Units per c.c. determined by animal experiment.
1	225	200
2	375	330
3	510	480

Effect of pH on the flocculation of a balanced mixture of diphtheria toxin and its concentrated antitoxin.

Using fresh diphtheria antitoxin, Schmidt (1930) noticed that the rate of flocculation of a balanced mixture of the toxin and the antitoxin depends to a great extent on the reaction of the medium. According to him at pH 4.5, pH 4.95, and

pH 9.5 the rate of flocculation was very slow, while between pH 5.55 and pH 8.0 it was constant and fairly rapid, and at pH 10.0 flocculation did not occur. Experiments were undertaken to ascertain how far the flocculation of concentrated diphtheria antitoxin depends on the reaction of the medium. The antitoxin used by us was concentrated by the method described in a previous paper (Ghosh and Ray, 1937). We have found that the rate of flocculation of a balanced mixture of the toxin and its concentrated antitoxin was the quickest between pH 6.6 and pH 8.0. Below pH 6.6 and above pH 8.0 the rate of flocculation became increasingly slow and at pH 10.0 flocculation did not occur. This shows that the range of pH within which the rate of flocculation is nearly constant and fairly rapid is shorter with concentrated than with unconcentrated diphtheria antitoxin.

Effect of pH on the flocculation of a balanced mixture of tetanus toxin and its concentrated antitoxin.

The antitoxin used in these experiments was concentrated by the method described in our previous paper (Ghosh and Ray, *loc. cit.*). The results obtained are recorded in Table II. It will be noticed that the rate of flocculation was nearly constant and fairly quick between pH 5.3 and pH 7.2. Below pH 5.3 and above pH 7.2 it became increasingly slow and at pH 9.8 flocculation did not occur.

TABLE II.

pH.	Time required for flocculation.	
	Hour.	Minutes.
4.6 ..	1	20
5.0 ..	0	46
5.3 ..	0	35
5.8 ..	0	35
6.2 ..	0	35
7.2 ..	0	41
8.0 ..	0	48
9.8 ..	a	a

Determination of the potency of concentrated diphtheria antitoxin by flocculation test and by animal experiment.

In a previous paper (Ghosh and Ray, *loc. cit.*) some results were recorded showing that there is a fair agreement between the values of the potency of the concentrated diphtheria antitoxin determined by the flocculation test and by animal experiment. Further work in this line was continued and the results so far obtained are recorded in Table III. It will be noticed that these results fully confirm the views expressed in our previous paper. It may be mentioned that each sample of concentrated antitoxin used was prepared from a mixture of fresh antiserum obtained from four or five immunized horses.

TABLE III.

Sample number of antitoxin.	Units per c.c. by flocculation test.	Units per c.c. by animal experiment.
1	1,070	1,000
2	2,000	2,000
3	750	840
4	1,860	1,800
5	1,490	1,600
6	700	750
7	1,000	1,150
8	1,400	1,550
9	2,250	2,200
10	1,380	1,600
11	1,500	1,600
12	1,600	1,800

Determination of the potency of concentrated tetanus antitoxin by the flocculation test and by animal experiment.

In a previous paper (Ghosh and Ray, *loc. cit.*) some preliminary results were recorded showing that the potency of tetanus antitoxin determined by the flocculation test agrees satisfactorily with that determined by animal experiment. The results of further experiments in this line are recorded in Table IV. Each sample of concentrated antitoxin used in these experiments was prepared from a mixture obtained by pooling the sera of five or six immunized horses. This procedure has the advantage that if the serum of one of these horses happen to be poorly avid, then in the mixture its effect will be greatly reduced owing to dilution

by the sera of the other horses. It will be noticed from the data recorded in Table IV that there is good agreement between the results obtained by flocculation test and those obtained by animal experiment. The results are expressed in American units.

TABLE IV.

Sample number of antitoxin.	Units per c.c. by flocculation test.	Units per c.c. by animal experiment.
1	1,500	1,600
2	1,000	1,000
3	800	750
4	1,000	1,000
5	700	820
6	850	900
7	900	1,000
8	530	600
9	1,206	1,200
10	1,000	1,150
11	860	750
12	1,250	1,200
13	1,100	1,000
14	918	980
15	675	750
16	884	960
17	990	1,080

CONCLUSION.

A method has been evolved by which diphtheria antitoxin concentrated by precipitation with sodium sulphate can be made to flocculate.

The rate of flocculation of a balanced mixture of diphtheria toxin and the concentrated antitoxin depends on the pH of the medium. It is nearly constant and fairly rapid between pH 6.6 and pH 8.0. At or above pH 10.0 flocculation does not occur.

The rate of flocculation of a balanced mixture of tetanus toxin and its concentrated antitoxin is nearly constant between pH 5·3 and pH 7·2. Below pH 5·3 and above pH 7·2 it becomes increasingly slow and above pH 9·8 flocculation does not occur.

In continuation of previous work further results which have been obtained show that the potency of the concentrated diphtheria antitoxin as well as that of the concentrated tetanus antitoxin determined by the flocculation test agrees well with that determined by experiments *in vivo*.

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THE DANYSZ PHENOMENA IN STAPHYLOCOCCAL TOXIN-ANTITOXIN REACTION.

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It was first observed by Danysz (1902) that a given amount of antitoxin which neutralizes a fixed quantity of ricin, when the latter is added to the former in one instalment, fails to do so when the addition is effected in several instalments at intervals of, say, 15 minutes. Later investigation showed that this phenomenon is fairly general and occurs with other pairs of toxin-antitoxin as well. Schmidt (1930) records that electrolyte-free mixtures of diphtheria toxin and antitoxin do not show this peculiarity in their reaction. Very little is as yet known regarding the mechanism of the reaction which causes this phenomenon. Bordet (1903) attempted to explain it on the basis of its close resemblance to the adsorption of a dyestuff by blotting-paper. It has been observed that when to a given volume of a dye solution a certain number of blotting-papers is added all at once, then the amount of dye adsorbed is less than when the same number of blotting-papers is added to the dye solution one after another at intervals of, say, 20 minutes. It follows from Bordet's picture that the ricin behaves like the blotting-paper and the antiricin like the dye, so that when the first portion of the toxin is added, it adsorbs more of the antitoxin than is necessary to neutralize it and the subsequent portions do not get enough antitoxin for their neutralization, with the consequence that the final mixture is toxic. If this view be correct, then the Danysz effect is associated with the inequality of distribution of the antitoxin among the different portions of the toxin, depending on the sequence of their addition and, with the attainment of uniformity of distribution of the antitoxin among the different portions of the toxin, the effect should disappear. From observations recorded by various authors (Cromwell, 1923 ; Ramon, 1930) it follows that toxin-antitoxin reaction is reversible at least to a great extent, if not completely so. Therefore, if a toxin-antitoxin mixture showing Danysz effect to start with is allowed to stand for

24 hours. The results obtained in one set of experiments in which the toxin was added to the antitoxin in exactly the same way as in experiment 4 in Table II are recorded in Table III:—

TABLE III.

Incubation period in hours before injection.	Observed intensity of reaction.
1	++
2	++
3	++
4	++
6	++
24	++

The Danysz effect in the reaction between staphylolysin and its antilysin.

It is well known that staphylococcal toxin can hæmolyse the red blood corpuscles of animals. This hæmolytic principle is called staphylolysin. The Danysz effect in staphylolysin-antilysin reaction was first observed by Sachs (1904). We have re-investigated the subject in greater detail. Samples of staphylococcal toxin and antitoxin were taken and their dilutions so adjusted by trial that 2 c.c. of the diluted antitoxin when mixed in one instalment with 2 c.c. of the diluted toxin the resulting mixture after 30 minutes' incubation at 37°C. produced very slight hæmolysis in 0.5 c.c. of a 0.4 per cent suspension of red blood corpuscles of rabbit. We first tried the effect of addition of 2 c.c. of the antitoxin in two or more instalments, at suitable intervals of time, to 2 c.c. of the toxin solution. The results are recorded in Table IV. The minus sign indicates no hæmolysis, the \pm sign denotes very slight hæmolysis, and +, ++, and +++ signs represent slight, moderate, and complete hæmolysis, respectively. In these experiments 0.5 c.c. of 4 per cent rabbit red blood corpuscles was added to 4 c.c. of the toxin-antitoxin mixture. Readings were taken three hours after the mixing of the red blood corpuscles. It will be noticed from the following data that when the antitoxin is added to the toxin in two or more instalments, at intervals of 20 minutes, the resulting mixture does not produce any hæmolysis. It therefore contains an

excess of antilysin. This excess was found to be equivalent to only 0.02 c.c. to 0.03 c.c. of the diluted toxin and hence constitutes 1 to 1.5 per cent of the total antitoxin used.

TABLE IV.

Volume of the diluted toxin taken = 2.0 c.c.

TIME OF ADDITION OF THE ANTITOXIN.			Observed intensity of the reaction.
12 a.m.	12-20 p.m.	12-40 p.m.	
2 c.c.	±
1 c.c.	1 c.c.	..	±
1 c.c.	..	1 c.c.	—
1 c.c.	0.5 c.c.	0.5 c.c.	—
0.5 c.c.	1 c.c.	0.5 c.c.	—

The effect of addition of toxin in different instalments to the antitoxin was next investigated. The other experimental details were exactly the same as described before. The results are recorded in Table V. It will be noticed from these data that when the toxin is added to the antitoxin in three instalments the resulting mixture is more toxic than when the addition is completed in two instalments.

TABLE V.

Volume of the diluted antitoxin taken = 2.0 c.c.

Experiment number.	TIME OF ADDITION OF THE ANTITOXIN.			Observed intensity of the reaction.
	11 a.m.	11-25 a.m.	11-50 a.m.	
1	2 c.c.	±
2	1 c.c.	1 c.c.	..	++
3	1 c.c.	..	1 c.c.	++
4	1 c.c.	0.5 c.c.	0.5 c.c.	+++
5	0.5 c.c.	1 c.c.	0.5 c.c.	+++

The effect of increasing the incubation period of toxin-antitoxin mixture on Danysz effect.

The effect of increasing the period of incubation of the toxin-antitoxin mixture after the addition and mixing of the last portion of the toxin was then investigated.

The toxin was added to the antitoxin in the manner indicated in experiments 2, 4, and 5 in Table V. The period of incubation was varied from 1 to 24 hours in each case. It was found that the hæmolytic power of the toxin-antitoxin mixture did not decrease during this period of incubation.

The results obtained in one typical set of experiments in which the toxin was added to the antitoxin in the manner described in experiment 5 in Table V are recorded in Table VI:—

TABLE VI.

Incubation period in hours before injection.	Observed intensity of reaction.
1	+++
2	+++
4	+++
6	+++
24	+++

CONCLUSION.

1. When a given amount of staphylococcus antitoxin is added to an equivalent quantity of the toxin in two or more instalments, at intervals of 20 minutes, the resulting mixture is neutral as measured by its dermo-necrotic effect.

2. If, however, the toxin is added to the antitoxin in two or more instalments, at intervals of 20 minutes, the resulting mixture is markedly toxic, as measured by the necrosis it produces on intradermal injection into rabbits. This property of the mixture does not appear to decrease with lapse of time.

3. When a given amount of staphylococcal antitoxin is added to an equivalent quantity of the toxin, at intervals of 20 minutes, the resulting mixture is completely non-toxic or slightly over-neutralized as measured by its hæmolytic properties.

4. On the other hand, when the toxin is added to an equivalent quantity of the antitoxin in two or three instalments, at intervals of 25 minutes, the mixture obtained is highly hæmolytic and its hæmolytic power does not decrease with lapse of time.

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THE RELATIVE IMMUNIZING VALUES OF DIFFERENT
FORMS OF ANTIRABIC VACCINE, AND THE
DURATION OF IMMUNITY IN
EXPERIMENTALLY
IMMUNIZED
ANIMALS.

BY

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THE comparison of results obtained with vaccine prepared from different strains of fixed virus, and with different preservatives, which forms the subject of this communication, was undertaken to determine whether other methods had any advantage over the method in general use in India, i.e., the use of a carbolized 5 per cent suspension of sheep's brain infected with the Paris strain of fixed virus.

MATERIALS AND METHODS.

The strains of fixed virus used in the experiments were :—

- (a) The Pasteur Institute, Paris, strain.
- (b) The Pasteur Institute, Kasauli, strain.

(c) Five strains including the above, viz., Paris, Kasauli, Jack-Human, Kasauli fixed virus 4, and Kasauli fixed virus 5.

The animals used in the preparation of the vaccines were rabbits and sheep and the preservatives were formalin and carbolic acid.

The experimental animals used in testing the efficacy of the various vaccines were monkeys, and the infective material used as test doses for this purpose was various freshly isolated strains of street virus.

The method of preparing the carbolized vaccines was that described by Shortt, Malone, Craighead and McGuire (1934), while formolized vaccines were prepared as follows:—

The brain and cord of a rabbit which had died of fixed virus infection were weighed.

This was ground into an emulsion with sand and a measured quantity of 9 per cent salt solution equal to half the weight of the brain and cord.

The emulsion was allowed to stand in the ice-chest overnight and then distilled water was added up to nine times the original quantity of salt solution.

This made a 20 per cent emulsion in normal saline.

Normal saline was then added to make a 5 per cent emulsion. This was formolized with 5 parts per 1,000 formalin. This technique was based on that of Hindle (1928) for the preparation of a yellow-fever vaccine.

The experimental routine followed was to immunize the animals, allow sixteen to seventeen days for the development of immunity, and then to infect them with the test street virus. Although the dosage was calculated on the dose for weight principle, each monkey being given the equivalent of the highest dose given to human beings bitten on the face, the monkeys were graded for size and, in each experiment, were actually all given a flat dose of the amounts stated.

EXPERIMENTS.

The results of experiments with each individual street virus are given separately in the tables below, along with any appropriate comments and these are followed by combined tables summarizing all the results. It may be pointed out that while each of the tables giving the results with a particular street virus gives a true indication of the interactions between the immune bodies and the street virus, the combined table gives a somewhat artificial picture since the street viruses used were different in each individual experiment and of varying virulence.

TABLE I.

Experiment 39.

Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	13	Daily flat dose of 2.5 c.c. for 14 days.	13	In muscles of neck.	0	1*	12	0
2. Formalin 5 per cent Paris strain rabbit vaccine.	13		12		1	3†	8	11.1
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	13		13		2	2‡	9	18.1
4. Carbolized 5 per cent Paris strain rabbit vaccine.	13		13		2	2‡	9	18.1
5. Carbolized 5 per cent polyvalent rabbit vaccine.	13		13		2	1§	10	16.6
6. Controls	13		10	0	3	76.9

* Passaged—not rabies.

† One passaged—not rabies. Two not passaged—no symptoms of rabies.

‡ Two passaged—not rabies.

§ One passaged—not rabies.

In this experiment all the vaccines gave a high degree of immunity and the street virus was of normal virulence.

TABLE II.
Experiment 41.
Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	12	Daily flat dose of 2.5 c.c. for 14 days.	12	In muscles of neck.	2	1*	9	18.1
2. Formalin 5 per cent Paris strain rabbit vaccine.	12		11		2	1*	8	20.0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	12		12		5	0	7	41.6
4. Carbolized 5 per cent Paris strain rabbit vaccine.	12		11		2	0	9	18.1
5. Carbolized 5 per cent polyvalent rabbit vaccine.	12		11		1	1*	9	10.0
6. Carbolized 1 per cent Paris strain sheep vaccine.	12	Daily flat dose of 0.28 c.c. for 14 days.	9		5	1†	3	62.5
7. Carbolized 5 per cent Kasauli strain sheep vaccine.	4	Daily flat dose of 5 c.c. for 7 days.	3		1	0	2	33.3
8. Controls	12		11	0	1	91.6

* One passaged—not rabies.

† Not passaged—died of pneumonia.

In this experiment, vaccines Nos. 3 and 6, especially the latter, were relatively ineffective against a street virus of high virulence as judged by the death rate among controls.

TABLE III.

Experiment 46.

Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	15	Daily flat dose of 2.5 c.c. for 14 days.	15	In muscles of neck.	2	0	13	13.3
2. Formalin 5 per cent Paris strain rabbit vaccine.	15		13		3	0	10	23.0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	15		15		3	0	12	20.0
4. Carbolized 5 per cent Paris strain rabbit vaccine.	15		14		2	0	12	14.2
5. Carbolized 5 per cent polyvalent rabbit vaccine.	15		13		1	0	12	7.6
6. Carbolized 1 per cent Paris strain sheep vaccine.	15	Daily flat dose of 0.28 c.c. for 14 days.	10		1	0	9	10.0
7. Carbolized 5 per cent Paris strain sheep vaccine.	15	Daily flat dose of 5 c.c. for 7 days.	14		1	0	13	7.1
8. Controls	15		7	0	8	46.6

The production of immunity in this series of animals was relatively poor considering the low virulence of the street virus.

TABLE IV.

Experiment 55.

Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies including deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	6	Daily flat dose of 2.5 c.c. for 14 days.	6	In muscles of neck.	2	1*	3	40.0
2. Formalin 5 per cent Paris strain rabbit vaccine.	6		5		0	0	5	0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	6		6		3	0	3	50.0
4. Carbolized 5 per cent Paris strain rabbit vaccine.	6		6		2	0	4	33.3
5. Carbolized 5 per cent polyvalent rabbit vaccine.	6		5		2	0	3	40.0
6. Carbolized 1 per cent Paris strain sheep vaccine.	6	Daily flat dose of 0.28 c.c. for 14 days.	5		2	0	3	40.0
7. Carbolized 5 per cent Paris strain sheep vaccine.	6	Daily flat dose of 5 c.c. for 7 days.	6		1	2†	3	25.0
8. Controls	6		6	0	0	100.0

* Passed—no rabies.

† One died of pneumonia. *N. B.* not seen. One passed—no result.

In this series, the street virus was of the highest virulence and this is reflected in the relative inefficiency of the vaccine. Vaccine No. 2 gave an extremely satisfactory result.

TABLE V.

Experiment 56.

Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	5	Daily flat dose of 2.5 c.c. for 14 days.	5	In muscles of neck.	1	0	4	20.0
2. Formalin 5 per cent Paris strain rabbit vaccine.	5		4		1	0	3	25.0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	5		5		1	0	4	20.0
4. Carbolized 5 per cent Paris strain rabbit vaccine.	5		5		1	0	4	20.0
5. Carbolized 5 per cent polyvalent rabbit vaccine.	5		4		0	0	4	0
6. Carbolized 1 per cent Paris strain sheep vaccine.	5	Daily flat dose of 0.28 c.c. for 14 days.	5		1	0	4	20.0
7. Carbolized 5 per cent Paris strain sheep vaccine.	5	Daily flat dose of 5 c.c. for 7 days.	5		0	0	5	0
8. Controls	5		2	0	3	40.0

The immunity produced in this series was poor considering the low virulence of the street virus.

COMBINED TABLE VI.

Pre-infectional immunization experiment.

Treatment.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	51	Daily flat dose of 2.5 c.c. for 14 days.	51	Intramuscularly in neck.	7	3*	41	14.5
2. Formalin 5 per cent Paris strain rabbit vaccine.	51		45		7	4†	34	17.0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	51		51		14	2‡	35	28.5
4. Carbolized 5 per cent Paris strain rabbit vaccine.	51		49		9	2‡	38	19.1
5. Carbolized 5 per cent polyvalent rabbit vaccine.	51		46		6	2‡	38	13.6
6. Carbolized 1 per cent Paris strain sheep vaccine.	38	Daily flat dose of 0.28 c.c. for 14 days.	29	Intramuscularly in neck.	9	1§	19	32.1
7. Carbolized 5 per cent Kasauli strain sheep vaccine.	4	Daily flat dose of 5 c.c. for 7 days.	3		1	0	2	33.3
8. Carbolized 5 per cent Paris strain sheep vaccine.	26		25		2	2¶	21	8.7
9. Controls	51		36	0	15	70.5

* 3 passaged—not rabies.

† 2 passaged—not rabies.

‡ One passaged—not rabies. One not passaged—died of pneumonia.

§ 2 passaged—not rabies. 2 not passaged—not rabies.

§ Not passaged—died of pneumonia.

The combined results of the five experiments in which eight different kinds of vaccines were used show that immunization of monkeys before infection has a very considerable saving effect. This is most marked in the case of carbolicized 5 per cent Paris strain sheep vaccine.

A comparison of the results of using *formalin* instead of *carbolic acid* as a preservative for the vaccine is made in Table VII:—

TABLE VII.

Preservative used in the vaccine.	Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
Formalin 0.2 per cent ..	102	1.75 grammes.	96	Intramuscularly in neck.	14	7	75	15.7*
Carbolic acid—0.6 per cent ..	183	1.75 grammes.	174		32	8	134	19.2*

* 38 animals immunized with carbolicized 1 per cent vaccine, which received only 0.392 gramme of brain material, have been excluded from the above figures.

This table shows a slight advantage in the use of formalin but this is not significant.

A comparison of the results obtained with the Kasauli, Paris, and polyvalent strains of fixed virus is made in Table VIII :—

TABLE VIII.

Strain of fixed virus.			Number of monkeys immunized.	Dosage.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes after infection.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
Kasauli	106	1.75 grammes.	105	Intramuscularly in neck.	22	5	78	22.0*
Paris	128	1.75 grammes.	119		18	8	93	16.2*
Polyvalent	51	1.75 grammes.	46		6	2	38	13.6*

*38 animals immunized with carbolized 1 per cent vaccine, which received only 0.392 gramme of brain material, have been excluded from the above figures.

This table shows some advantage in the cases of the Paris and polyvalent vaccines, but it must be remembered that the latter also contains the Paris strain of fixed virus.

THE DURATION OF IMMUNITY IN MONKEYS EXPERIMENTALLY IMMUNIZED AGAINST RABIES.

As a considerable number of monkeys which had been used in various experiments in connection with antirabic immunization was found to be available, it was decided to utilize these in an attempt to determine the presence and degree of immunity existing six months and more after the last immunizing dose.

The monkeys available were those which had escaped the results of infection with street virus in the experiments previously described and had, therefore, each been through the following process of immunization :—

(a) A course of vaccine containing killed fixed virus.

(b) A single inoculation with living and fully virulent street virus.

The results of the individual experiments are given separately in Tables IX to XI followed by Table XII in which the results of the five experiments are combined.

TABLE IX.

Experiment 45.

Duration of immunity in immunized animals.

Nature of treatment.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	10	In muscles of neck.	2	0	8	20.0
2. Formalin 5 per cent Paris strain rabbit vaccine.	8		0	0	8	0
3. Carbolyzed 5 per cent Kasauli strain rabbit vaccine.	9		1	1*	7	12.5
4. Carbolyzed 5 per cent Paris strain rabbit vaccine.	7		0	0	7	0
5. Carbolyzed 5 per cent polyvalent rabbit vaccine.	10		2	0	8	20.0
6. Controls	12		12	0	0	100.0

* Passed—*not* rabies.

Note.—Animals surviving from experiment 39 were used.

Date of commencement of immunization for experiment 39—24th June, 1931.

Date of completion of immunization for experiment 39—7th July, 1931.

Date of 1st infection with street virus 1 for experiment 39—24th July, 1931.
6½ months.

Date of 2nd infection with street virus 2 for experiment 45—10th February, 1932.
6½ months.

This experiment is less satisfactory as the infecting street virus was of low virulence. Again, however, the Paris virus shows up well, even when the vaccine prepared from it is used as a 1 per cent emulsion.

COMBINED TABLE XII.

Duration of immunity in immunized animals.

Nature of treatment.	Number of monkeys infected with street virus.	Method of infection.	Died of rabies.	Died of other causes.	Escaped infection.	Percentage of deaths due to rabies excluding deaths from other causes.
1. Formalin 5 per cent Kasauli strain rabbit vaccine.	26	In muscles of neck.	2	1	23	8.0
2. Formalin 5 per cent Paris strain rabbit vaccine.	24		0	0	24	0
3. Carbolized 5 per cent Kasauli strain rabbit vaccine.	23		2	1*	20	9.0
4. Carbolized 5 per cent Paris strain rabbit vaccine.	24		0	1*	23	0
5. Carbolized 5 per cent polyvalent rabbit vaccine.	25		4	0	21	16.0
6. Carbolized 1 per cent Paris strain sheep vaccine.	7		0	1†	6	0
7. Carbolized 5 per cent Paris strain sheep vaccine.	8		0	0	8	0
8. Controls	34		24	0	10	70.5

* Passaged—not rabies.

† Passaged—no results.

Note.—Animals surviving from experiments 39, 41, 55, and 56 were used.

Combined Table XII shows that a very high degree of immunity is present six months or more after immunization by all the vaccines used. The degree of immunity is much greater than in the experiments where monkeys were tested a few weeks after immunization, as a comparison with the first series of experiments

described above will show. The reality of this greater immunity is also evidenced by the fact that the virulence of the infecting viruses is the same as in the previous experiments.

We are inclined to attribute this high degree of immunity to the fact that the monkeys in this series had already (in previous experiments) received and survived a dose of live and virulent street virus which conferred a degree of immunity not readily obtainable with dead vaccines.

SUMMARY AND CONCLUSIONS.

1. Antirabic killed vaccine prepared with different strains of fixed virus and preserved with either formalin or carbolic acid produces a considerable degree of immunity.

2. Formalin and carbolic acid have no special advantages, one over another, as preservatives for the vaccine.

3. The Paris strain of fixed virus appears to be a better antigen than the other strains experimented with.

4. A very high degree of immunity exists six months or more after immunization when the production of the latter has involved the use of one dose of live and virulent street virus.

REFERENCES.

- HINDLE, E. (1928) *Brit. Med. Jour.*, 9th June, pp. 976-977.
SHORTT, H. E., MALONE, R. H., CRAIGHEAD, A. C., and MCGUIRE, J. P. (1934), *Ind. Med. Res. Memoirs* No. 28, January, pp. 1-24.

TABLE I.

Table showing number of parasites per 100 fields of the 1/12th oil immersion lens and eyepiece No. 4.

DAY OF INFECTION.																				
Animal.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Dog 45 ..	22	26	10	4	60	29	26	5	9	6	384	129	613	38	72	332	46	241	362	Died.
Dog 46 ..	4	84	158	294	403	502	558	674	1,023	72	46	258	842	49	173	191	784	Died

infections with *B. canis*, the dogs were not known to be 'clean' dogs, and tick-proof kennels were not used, these experiments would appear to lose much of their value since in most parts of India the ground is infested with ticks of various species and *B. gibsoni* is not an uncommon parasite in northern India.

EXPERIMENTS IN THE PRESENT SERIES.

Materials.—The dogs used were all indigenous, i.e., born in India, although not necessarily 'pie' dogs. Dogs collected from the bazaars in Madras, even when mere puppies, are infected with *B. canis* in the proportion of about 90 per cent. Out of over 400 dogs collected from the same areas and from the Madras lethal chamber not one showed infection with *B. gibsoni*. Now, as mentioned above, we have found that once infection with *B. gibsoni* is established the parasites are always present in the blood although the numbers may vary from day to day in the same dog, so that unless extremely rare it would have been in evidence in some at least of the dogs. In illustration of this point we give below parasite counts made daily in two dogs from the establishment of infection up to death of the animal.

A certain number of our animals were pregnant bitches imported from the hill station of Coonoor. These gave us clean puppies with which to work.

The ticks employed in our experiments were of one species only, viz., *H. bispinosa*. The ticks were originally collected from jackals and from these a breeding stock was established which was utilized in all experiments. This tick fed readily on dogs and jackals and clean ticks were available throughout the investigation. A species of *Hyalomma* was found on one occasion on a young jackal and three times was encountered in jackal dens but was definitely rare. *H. bispinosa*, on the other hand, swarmed on the jackals and in their dens.

Methods.—During the conduct of these experiments no species of ticks other than *H. bispinosa* was brought into the laboratory. This was to avoid any possible mixing up of species which might occur in the earlier stages of the ticks. The dogs used in the experiments were 'de-ticked', washed in an insecticide, and accommodated in cages previously sterilized and mounted on tables which stood in disinfectant solution. The cages themselves, in the case of those dogs which were used for feeding ticks, were entirely enclosed in muslin bags which were changed daily and from which engorged ticks were collected after they had dropped from the dogs.

The procedure in any individual experiment was as follows:—

Clean laboratory-bred ticks, larvæ, nymphs, or adults were fed upon a dog infected with *B. gibsoni*. The engorged ticks, after dropping, were collected and kept until they moulted into the next stage. If the stage to be experimented with was that subsequent to the one fed, the newly moulted ticks were placed on a clean dog or jackal with the object of infecting the latter, if possible. If a later stage was to be the subject of experiment, the intermediate feed was also on a normal animal. As examples, ticks fed as larvæ on an infected dog could be used in transmission experiments as (a) nymphs and (b) adults (with one intermediate feed), and

ticks fed as adults could be used in transmission experiments as larvæ and nymphs (hereditary transmission through the egg).

EXPERIMENTS.

TABLE II.

Table showing experimental infection of dogs and jackals with B. gibsoni, using the jackal tick Hæmaphysalis bispinosa.

Experimental animal and number.	Stage of ticks infected by feeding on an infected dog.	Stage of same ticks used for transmission experiments.	Result.	Incubation period.	REMARKS.
Dog 26 ..	Larvæ	Nymphs	Negative	..	That the dog was not immune was proved by its subsequent infection by syringe four months later.
„ 38 ..	Adults	Larvæ	„
„ 40	„	„	Positive	19 days	Larvæ from ten adults used.
„ 41 ..	Larvæ	Nymphs	Negative	..	Larvæ used for this experiment and the adults used for the previous experiment were fed on the same infected dog at the same time.
„ 48 ..	Adults	Larvæ	„
„ 49 ..	„	„	„	..	That the dog was not immune was proved by its subsequent infection by syringe two months later.
„ 54 ..	Larvæ	Nymphs	Negative
„ 55 ..	Adults	Larvæ	Positive	14 days	Larvæ from fifteen adults used.
„ 60 ..	„	„	„	12 „	Larvæ from eight adults used.
„ 79 ..	„	„	„	12 „	Larvæ from five adults used.
„ 93 ..	„	Nymphs	„	22 „	The larvæ were fed on an uninfected dog and the resulting nymphs were used for this experiment.

RED CELLS, HÆMOGLOBIN, COLOUR INDEX, SATURATION INDEX, AND VOLUME INDEX STANDARDS.

Part I.

NORMAL INDIAN MEN: A STUDY BASED ON THE EXAMINATION OF 121 MEN.

BY

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ABOUT eight years ago when we were engaged in the study of the blood condition in sprue, we came up against the fact that we had no normal standards for Indians with which we could compare our pathological findings. The situation did not seem to be much better for Europeans either. Osgood (1926) had drawn attention to the fact that the figures given in textbooks as normal standards had no adequate basis. He stated: 'The figures for average normal counts (5 millions for men and 4·5 millions for women) were adopted a long time ago, and have been quoted from one textbook to another without proper confirmation. Few physicians are aware that these figures were based on examination of a small number of bloods. Vierord (1852) and Welcher (1854), each of whom examined the blood of only two persons, are responsible for these figures (according to Bie and Möller)'. The position regarding hæmoglobin was no better. Brown and Rowntree (1925) stated 'the normal standard of hæmoglobin is as yet unsettled, this is somewhat surprising

in a substance so important'. Haden (1933) called attention to the number of widely different hæmoglobin figures used as 100 per cent by various authors and instrument makers (e.g., 13.8 g., 14.00 g., 15.00 g., 15.36 g., 15.80 g., 16.00 g., 16.90 g., and 17.20 g.).

When we began work in 1929 the only reliable figures available to us were those of Osgood (1926). He, employing accurately standardized methods, had made determinations on 137 young American men between the ages of 19 and 30 years. In our study of 121 healthy Indian males, we have followed him closely except for hæmoglobin determination, as we will explain below. A short summary of our results was published in the report of the Haffkine Institute for the year 1929 (Sokhey, 1929). Since the completion of our work other studies have been published. To these we shall refer in the body of our paper.

METHODS.

All tests were made on venous blood as suggested by Osgood (1926). For each examination 10 c.c. of blood were drawn from a vein and quickly introduced into a 50 c.c.-Erlenmeyer flask containing 20 mg. of finely powdered neutral potassium oxalate and thoroughly mixed by whirling the flask while keeping it pressed against a flat surface. No tourniquet was used while drawing blood, slight pressure of the hand was found quite sufficient. This precaution is necessary to avoid any undue congestion of the limb.

The use of oxalated blood for various determinations is important. It guarantees exactly the same concentration of red cells in the portion of blood used for hæmoglobin estimation or for red cell volume determination, as in the portion used for the red cell count. Using separate drops from the ear or finger does not give us any such assurance of uniformity (Osgood, 1926).

Red cell count.—Levy-Hausser counting chambers with Neubauer rulings and Trenner pipettes were used throughout. Both the chambers and pipettes used had been tested by the American Bureau of Standards.

The flask containing the sample was thoroughly whirled and a little blood taken on a paraffined watch-glass to charge the pipettes. Hayem's solution was used as the diluent. At least two counts were made in each case and an average taken. Whenever the duplicates differed by more than 2 per cent a fresh dilution was made and the counts repeated.

Hæmoglobin.—The determination of hæmoglobin presented many difficulties. We tried the method described by Osgood and Haskins (1923), in which hæmoglobin is converted into acid hematin and compared against an artificial standard, and found that the tint of the acid hematin of some samples differed so definitely from the tint of the standard that a perfect colour match was difficult. Consequently we made all our determinations by the oxygen capacity method of van Slyke and Neill (1924). The figures given in our tables are those obtained by this method. The oxygen capacity method though very accurate is not convenient for clinical work. Therefore during our study we continued to investigate various other methods. The results of these comparative determinations in detail will be published separately, but we like to state here that in our hands Newcomer's (1919, 1923) method gave results closest to the figures obtained by van Slyke's oxygen

capacity method. In Newcomer's method the hæmoglobin is converted into acid hematin and is compared against a specially prepared yellow glass-disc in a colorimeter. Messrs. Bausch and Lomb also supply a special blue glass-filter, which when placed over the upper lens of the eyepiece of the colorimeter, cuts out those parts of the spectrum which otherwise render a perfect colour match difficult. We give below in Table I comparative figures obtained in 20 cases by these two methods:—

TABLE I.

Comparison of estimations of hæmoglobin by Newcomer's method and by van Slyke's method.

Number.	Van Slyke, grammes per 100 c.c.	Newcomer, grammes per 100 c.c.	Number.	Van Slyke, grammes per 100 c.c.	Newcomer, grammes per 100 c.c.
1	16.21	16.16	12	10.81	11.31
2	12.15	12.51	13	12.53	13.75
3	15.70	16.20	14	12.27	12.91
4	15.66	16.28	15	16.86	17.59
5	8.84	9.52	16	12.54	12.92
6	14.18	14.26	17	11.59	11.48
7	13.27	13.75	18	11.38	11.10
8	14.72	14.90	19	12.69	12.28
9	13.78	14.26	20	14.65	14.90
10	13.69	13.80	AVERAGE ..	13.18	13.51
11	10.00	10.30			

The above results are the average of at least two determinations. But if the difference between any duplicates was found to be greater than 1 per cent, determinations were repeated.

Cell volume.—Hæmatocrit tubes for this determination were made from 5-c.c. pipettes graduated to the tip. The tip was sealed in the flame and the pipette cut off to leave a length of about 12 cm. These pipettes were then carefully recalibrated. The inside diameter of our pipettes was about 0.7 mm. Pipettes were fitted into metal centrifuge tube holders with rubber corks as described by Osgood (1926).

About 3 c.c. of thoroughly mixed oxalated sample were introduced into the hæmatocrit tube described above and centrifuged at a speed of about 4,500 revolutions per minute. We used International electric centrifuge, size 1. After centrifuging for a period of 30 minutes the volume of packed red cells was read and the tube again centrifuged for five-minute periods until successive readings showed no change in volume.

Suitable ready-made hæmatocrit tubes can now be obtained from Messrs. Arthur H. Thomas Co. (Osgood, 1935).

The shrinkage of the cells caused by potassium oxalate used as an anticoagulant and the effect of the speed and duration of centrifugation on cell volume is discussed in the body of the paper. In this determination we have followed Osgood (1926) closely, so that our determinations are exactly comparable to his, the only carefully worked out figures available at the time we started our work.

SUBJECTS EXAMINED.

One hundred and twenty-one men were examined. These men were mostly medical students from a neighbouring Medical College and were between 19 and 30 years of age. All our subjects belonged to the Bombay Presidency. Some of them were vegetarians, while others were on mixed diet. The diet of the two groups was much the same except that those on mixed diet consumed a little mutton or eggs now and then. Both groups consumed milk. The figure for total nitrogen excretion in 24-hour samples of urine seldom exceeded 7 g.

TABLE II.

Blood findings in one hundred and twenty-one normal young men.

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmoglobin, grammes per 100 c.c.	Hæmoglobin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
1	21	M	4.32	13.65	15.80	36.84	42.64	1.05	1.04	1.01
2	29	M	5.02	14.80	14.74	41.58	41.42	0.98	1.01	0.97
3	27	V	4.84	14.87	15.36	40.41	41.76	1.02	1.02	1.00
4	25	V	4.95	15.68	15.84	45.15	45.61	1.05	1.12	0.94
5	26	M	5.33	15.82	14.84	48.01	45.04	0.99	1.10	0.90
6	22	M	4.90	15.19	15.50	41.99	42.85	1.03	1.05	0.98
7	22	M	5.03	12.98	12.90	39.39	39.15	0.86	0.96	0.89
8	30	V	4.60	14.18	15.41	41.33	44.82	1.03	1.10	0.93
9	24	V	4.81	14.77	15.35	41.38	43.02	1.02	1.05	0.97
10	29	M	4.65	14.98	16.11	39.86	42.86	1.07	1.05	1.02
11	24	M	5.07	14.98	14.77	40.07	39.51	0.98	0.97	1.02

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
12	29	V	4.25	14.95	17.59	40.70	47.88	1.17	1.17	1.00
13	30	V	5.58	15.96	14.30	41.75	37.42	0.95	0.92	1.04
14	27	V	5.23	16.23	15.52	41.54	39.72	1.03	0.97	1.06
15	29	V	5.27	15.02	14.25	38.78	36.81	0.95	0.90	1.05
16	29	V	5.12	16.45	16.06	41.24	40.27	1.07	0.99	1.08
17	30	V	4.91	15.68	15.97	41.53	42.35	1.06	1.04	1.02
18	26	V	5.04	16.90	16.77	43.02	42.68	1.12	1.05	1.07
19	27	M	5.11	15.23	14.90	39.05	38.21	0.99	0.94	1.06
20	29	V	5.21	15.63	15.00	42.43	40.71	1.00	1.00	1.00
21	26	M	5.12	15.37	15.01	37.90	37.01	1.00	0.91	1.10
22	25	V	5.33	14.83	13.91	35.95	33.73	0.93	0.83	1.12
23	24	M	5.35	15.17	14.18	38.03	35.54	0.94	0.87	1.08
24	28	M	5.27	15.46	14.67	44.92	42.62	0.98	1.04	0.94
25	29	V	5.12	14.74	14.39	39.40	38.42	0.96	0.94	1.02
26	27	M	5.26	14.26	13.56	42.41	40.31	0.90	0.99	0.92
27	23	M	5.20	15.35	14.76	40.41	38.86	0.98	0.95	1.03
28	22	V	5.07	15.31	15.10	41.46	40.89	1.00	1.00	1.00
29	21	V	4.07	14.29	17.56	40.64	49.92	1.17	1.22	0.96
30	21	M	4.58	13.71	14.97	34.32	37.46	1.00	0.92	1.08
31	22	V	5.20	15.10	14.52	42.58	40.94	0.96	1.00	0.96
32	21	V	4.86	15.45	15.90	38.56	39.68	1.06	0.97	1.09
33	22	M	5.04	15.15	15.03	43.64	43.30	1.00	1.06	0.94
34	23	V	5.09	14.79	14.53	41.15	40.41	0.97	0.99	0.98
35	24	M	5.19	16.36	15.76	44.73	43.09	1.05	1.06	0.99
36	25	M	5.39	15.84	14.69	45.05	41.79	0.98	1.02	0.96

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmoglobin, grammes per 100 c.c.	Hæmoglobin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
37	27	V	5.46	16.45	15.06	46.56	42.64	1.00	1.04	0.96
38	26	M	5.09	14.94	14.68	41.12	40.40	0.98	0.99	0.99
39	21	V	5.22	16.12	15.44	38.91	37.27	1.03	0.91	1.13
40	25	V	5.04	16.64	16.51	45.52	45.16	1.10	1.11	0.99
41	22	V	5.00	16.27	16.27	39.93	39.93	1.08	0.98	1.11
42	23	V	5.47	15.44	14.11	44.28	40.48	0.94	0.99	0.95
43	24	V	5.35	15.62	14.60	39.67	37.08	0.97	0.91	1.07
44	25	V	5.18	15.90	15.35	43.60	42.09	1.02	1.03	0.99
45	26	V	4.89	14.04	14.36	41.05	41.98	0.96	1.03	0.93
46	28	M	5.55	16.31	14.69	39.56	35.65	0.98	0.87	1.12
47	28	M	5.63	16.47	14.63	46.61	41.40	0.97	1.01	0.96
48	27	V	4.95	13.27	13.40	40.09	40.49	0.89	0.99	0.90
49	23	V	4.57	14.00	15.32	36.84	40.31	1.02	0.99	1.03
50	29	V	4.24	13.40	15.80	32.47	38.29	1.05	0.94	1.12
51	28	M	5.87	17.63	15.02	49.02	41.77	1.00	1.02	0.98
52	25	V	4.76	14.10	14.81	39.61	41.61	0.99	1.02	0.97
53	21	V	5.28	16.66	15.78	44.52	42.17	1.05	1.03	1.02
54	25	M	4.90	15.49	15.81	39.82	40.63	1.05	1.00	1.06
55	25	V	5.15	15.50	15.05	37.28	36.19	1.00	0.89	1.13
56	22	V	4.21	13.21	15.69	38.75	46.03	1.04	1.13	0.93
57	24	V	5.78	17.28	14.95	46.91	40.58	0.99	0.99	1.00
58	26	M	4.69	14.71	15.68	37.61	40.10	1.03	0.98	1.06
59	26	M	5.33	15.74	14.77	46.84	43.94	0.98	1.08	0.91
60	22	M	5.45	16.35	15.00	46.87	42.99	1.00	1.05	0.95
61	22	V	4.93	15.58	15.80	40.29	40.86	1.05	1.00	1.05

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Satura-tion index.
62	23	V	5.95	16.47	13.84	42.97	36.11	0.92	0.88	1.04
63	21	M	5.23	16.17	15.46	44.80	42.83	1.03	1.05	0.98
64	24	V	5.58	16.80	15.05	46.41	41.50	1.00	1.02	0.98
65	19	M	5.20	15.49	14.89	44.25	42.55	0.99	1.04	0.95
66	22	V	5.02	14.20	14.14	41.92	41.76	0.94	1.02	0.92
67	22	M	5.64	17.69	15.68	46.22	40.98	1.04	1.00	1.04
68	26	M	5.25	16.52	15.73	43.55	41.48	1.05	1.02	1.03
69	22	V	5.03	15.48	15.39	36.28	36.06	1.02	0.88	1.16
70	23	M	5.51	17.12	15.54	46.55	42.24	1.03	1.03	1.00
71	27	M	5.58	16.40	14.70	43.74	39.20	0.98	0.96	1.02
72	24	V	5.46	15.97	14.62	40.00	36.63	0.97	0.90	1.09
73	26	V	5.11	15.46	15.13	41.70	40.82	1.01	1.00	1.01
74	22	V	5.04	15.95	15.82	42.65	42.32	1.05	1.04	1.02
75	28	M	5.61	16.32	14.55	44.24	39.43	0.97	0.97	1.00
76	29	V	4.62	14.93	16.16	41.37	44.78	1.07	1.10	0.98
77	24	V	5.30	14.94	14.09	42.63	40.22	0.94	0.98	0.95
78	29	V	5.61	16.66	14.85	46.74	41.66	0.99	1.02	0.97
79	25	V	4.99	15.46	15.49	43.11	43.19	1.03	1.06	0.97
80	25	V	4.89	15.65	16.00	42.43	43.38	1.06	1.06	1.00
81	25	V	5.23	15.64	14.95	43.40	41.50	0.99	1.02	0.98
82	29	V	5.29	16.33	15.44	42.47	40.14	1.03	0.98	1.04
83	23	V	4.99	15.38	15.41	43.17	43.25	1.02	1.06	0.97
84	21	V	5.16	14.96	14.50	41.27	39.99	0.96	0.98	0.98
85	29	V	5.26	15.29	14.53	36.29	34.49	0.97	0.84	1.14
86	30	V	5.45	16.29	14.95	42.54	40.86	0.99	0.96	1.04

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
87	29	V	5.70	17.06	14.97	46.69	40.96	0.99	1.00	0.99
88	22	V	5.68	16.07	14.15	44.04	38.77	0.94	0.95	0.99
89	30	V	5.35	14.57	13.62	38.05	35.55	0.91	0.87	1.04
90	26	V	4.20	13.88	16.52	40.38	48.07	1.10	1.18	0.93
91	21	V	4.56	15.51	17.01	40.32	44.21	1.13	1.08	1.04
92	20	V	4.79	15.26	15.93	42.00	43.84	1.06	1.07	0.99
93	28	V	4.45	15.38	17.28	40.00	44.93	1.15	1.10	1.04
94	27	M	5.48	15.40	14.05	44.18	40.30	0.93	0.99	0.95
95	20	V	5.22	16.14	15.46	43.56	41.73	1.03	1.02	1.01
96	25	V	5.32	15.35	14.43	38.13	35.83	0.96	0.88	1.09
97	22	V	5.18	16.17	15.61	42.03	40.57	1.04	0.99	1.04
98	20	V	4.93	14.36	14.56	41.05	41.64	0.97	1.02	0.95
99	26	V	4.86	14.78	15.21	39.88	41.04	1.01	1.00	1.01
100	29	V	4.99	15.01	15.04	41.25	41.33	1.00	1.01	0.99
101	19	V	4.85	14.77	15.23	41.90	43.20	1.01	1.06	0.96
102	28	V	5.28	16.78	15.89	44.94	42.56	1.06	1.04	1.01
103	30	M	5.10	15.46	15.17	41.42	40.61	1.01	0.99	1.01
104	19	M	5.24	14.21	13.56	41.12	38.28	0.90	0.94	0.96
105	26	M	5.62	15.97	14.21	46.99	41.81	0.94	1.02	0.92
106	25	M	5.21	15.28	14.66	44.02	42.25	0.98	1.03	0.94
107	24	M	5.14	14.40	14.01	41.83	40.68	0.93	1.00	0.93
108	29	V	4.89	14.20	14.52	38.36	39.22	0.97	0.96	1.00
109	26	M	4.35	12.75	14.66	36.48	41.93	0.97	1.03	0.95
110	29	V	5.15	15.14	14.70	40.42	39.24	0.98	0.96	1.02
111	26	V	4.74	13.67	14.42	38.59	40.70	0.96	1.00	0.96

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II—concl'd.

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coeffi- cient.	Cell volume, c.c. per 100 c.c.	Cell volume coeffi- cient.	Colour index.	Volume index.	Satura- tion index.
112	24	V	5.17	15.82	15.30	42.91	41.50	1.02	1.02	1.00
113	29	V	4.98	15.24	15.30	41.82	41.99	1.02	1.03	0.99
114	26	M	5.79	15.96	13.78	43.49	37.56	0.92	0.92	1.00
115	29	V	5.19	15.74	15.16	44.54	42.91	1.01	1.05	0.96
116	26	V	4.51	14.60	16.19	38.32	42.67	1.08	1.04	1.03
117	26	V	4.41	14.69	16.66	39.24	44.49	1.11	1.09	1.02
118	29	V	5.59	15.53	13.89	42.67	38.17	0.92	0.93	0.99
119	20	V	4.78	14.70	15.38	40.20	42.06	1.02	1.03	0.99
120	19	V	5.47	14.62	13.36	43.05	39.34	0.89	0.96	0.92
121	20	M	5.85	15.16	12.96	43.99	37.59	0.86	0.92	0.94
AVERAGE	25	..	5.11	15.37	15.03	41.72	40.83	1.00	1.00	1.00

In the diet column, V stands for vegetarian diet and M for mixed diet.

RED CELL COUNTS.

Fig. 1 shows the frequency distribution of red cell counts in our subjects—121 men. Our average in 121 male subjects between the ages of 19 and 30 is 5,111,000. The values range from 4.07 to 5.95 millions. The mean (5.111 ± 0.023) and the median (5.150 ± 0.029) are practically the same. The standard deviation is 0.377 ± 0.016 and the coefficient of variation is 7.365 ± 0.321 . The significant variation is 4.73–5.49, 71 per cent of our subjects fall in this range.

The usual figure given in European and American textbooks for the average normal count for men is 5 millions. But Osgood (1926), as the result of examination of 137 men in West United States, found the average to be 5,390,000, while Wintrobe and Miller (1929) obtained an average of 5,850,000 based on a study of 100 subjects in South United States. Taking all the available accurate figures for Europe and America, Wintrobe (1933) found the average for 477 subjects to be 5,430,000.

Napier and Das Gupta (1935) obtained an average figure of 5·362 million cells for 50 healthy Indian males and later (1936) obtained an average of 5·533 millions for another series of 30 healthy Indian males. These individual averages as well as the combined average of the two series, 5·425 millions, are much higher than our figure. In the absence of detailed data of their individual determinations, it is

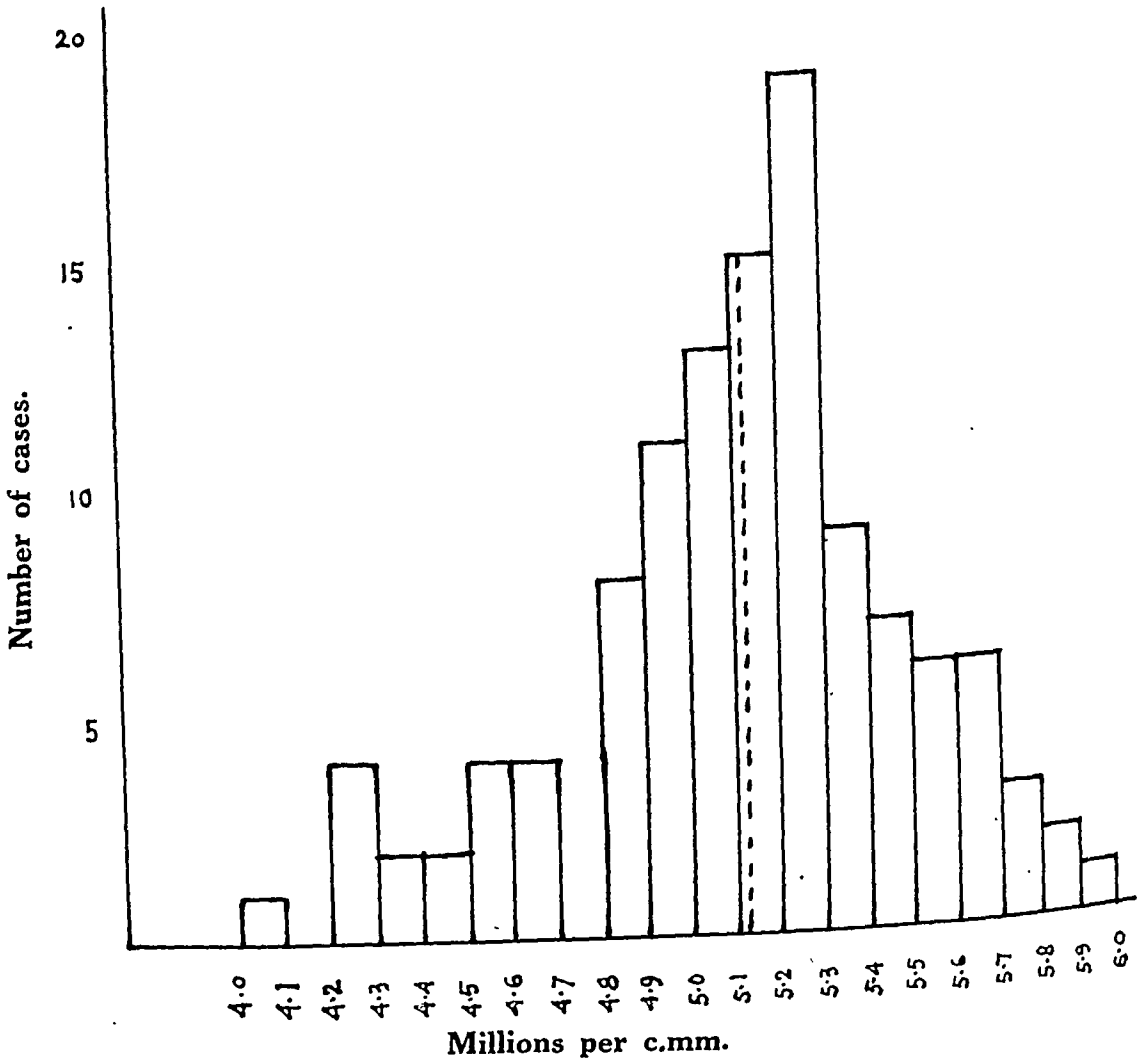


FIG. 1. Red cell counts in 121 men.

not possible to assess the significance of their higher averages. There is another difficulty. Their results cannot be compared with ours nor with those of Osgood and Wintrobe because the age range of their subjects (25 to 45 years) is different from that adopted by us, Osgood and Wintrobe.

HÆMOGLOBIN CONTENT.

We express our hæmoglobin figures as so many grammes per 100 c.c. of blood. Our average in 121 male subjects is 15·37 g. hæmoglobin corresponding to an oxygen capacity of 20·60 c.c. per cent. Fig. 2 gives the frequency distribution of hæmoglobin in all our subjects. The values ranged from 12·75 g. to 17·69 g. The mean ($15\cdot367 \pm 0\cdot059$) and the median ($15\cdot336 \pm 0\cdot073$) are very close. The standard deviation is $0\cdot955 \pm 0\cdot041$ and the coefficient of variation is $6\cdot221 \pm 0\cdot272$. The significant variation is 14·41 to 16·32; 72 per cent of our cases are within this range.

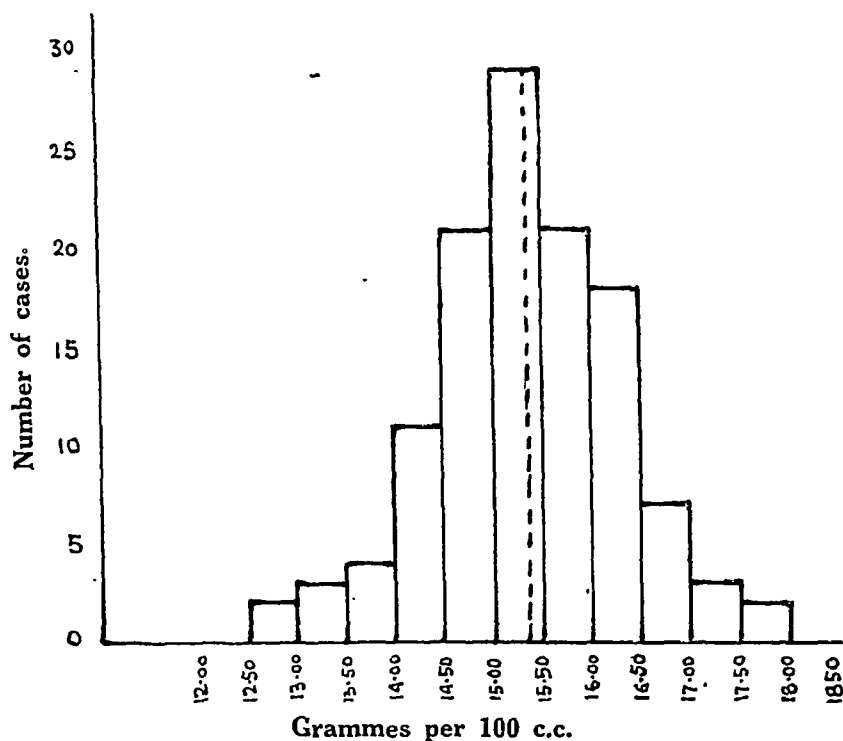


FIG. 2. Total hæmoglobin in 121 men.

Osgood (1926) found in literature reliable hæmoglobin estimations reported for only 70 males between 19 and 30 years of age. The average of these 70 estimations was 16·27 g. Osgood's own average for 137 men of the same age group is 15·76 g. Later Wintrobe and Miller (*loc. cit.*) obtained an average of 15·87 g., corrected later by Wintrobe (1929) to 17·0 g., for 100 men between 19 and 30 years. For Indian males the only figures available at the time of writing are those of Napier and Das Gupta (1935, 1936). For a group of 50 men (1935) they obtained an average of 14·77 g., and for another group of 30 men (1936) their average was 15·70 g. The combined average of these two groups is 15·12 g. It is to be noticed, however, that

age composition of the two groups studied by Napier and Das Gupta is different from the age range adopted by us, Osgood and Wintrobe. For this reason and because of absence of details of individual data we can not usefully compare Napier and Das Gupta's figures with ours.

We wish to particularly stress the value of our hæmoglobin figures. This is the first large series reported (Sokhey, *loc. cit.*) in which all the determinations were made by the van Slyke oxygen capacity method. Since then two other series of hæmoglobin determinations with the van Slyke apparatus have been reported by Haden (1933). Haden in a series of 70 men obtained an average of 15·34 g., and Dill (1930) (quoted by Haden, 1933) an average of 15·38 g. in an examination of 40 men.

Haden and Dill's subjects were from the north-east of the United States of America. It is to be noted that though our subjects were Indian (Bombay Presidency) and had widely different dietetic habits and lived under totally different climatic conditions, the average hæmoglobin figure is approximately the same; the three figures being 15·34 g., 15·38 g., and 15·37 g. The similarity of these figures is very striking and it would be profitable to pursue this point further by making a large series of determinations in different places with the van Slyke apparatus.

Price-Jones (1931) examined blood of 100 healthy men in London with the Haldane blood gas apparatus and obtained a mean value of 14·5 g. per 100 c.c. of blood. This figure is markedly lower than the American average obtained by Haden and Dill with the van Slyke oxygen capacity method. On comparison of the two methods Price-Jones came to the conclusion that the difference between the two methods is slight and quite inadequate to explain the difference between his figures and the American ones. Further neither altitude, nor age, nor standard of living would explain the difference, because the two groups examined in London and Boston were socially similar and were of the same age and lived at sea-level. He is inclined to attribute the difference to the extensive motoring habit of the Americans which tends to cause a slow chronic carbon monoxide poisoning, leading to a relative polycythæmia. But this surely is not the whole explanation, because our hæmoglobin value is as high as the American one, though our subjects were not exposed to any appreciable risk of carbon monoxide poisoning. There are relatively few motor cars in use in Bombay.

HÆMOGLOBIN COEFFICIENT AND COLOUR INDEX.

The determination of the colour index necessitates a comparison of the hæmoglobin content of a given individual to a normal standard and expresses the ratio of the amount of hæmoglobin per red cell of the individual to that of the average normal. The normal standard is taken to be the average number of grammes of hæmoglobin per 100 c.c. of blood calculated to a count of 5 million red cells per c.mm. in the average healthy person of the same age and sex group. Osgood (1926) suggests the term 'hæmoglobin coefficient' to replace the cumbersome expression 'the number of grammes of hæmoglobin per 100 c.c. of blood calculated to a red cell count of 5 millions per c.mm.'

The actual hæmoglobin content in grammes per 100 c.c. of blood of the individual is converted into a percentage of the normal hæmoglobin coefficient

of the same age and sex group. Also the red cell count of the individual is converted into a percentage of a count of 5 millions taken as 100 per cent. The colour index is then calculated by dividing the percentage of hæmoglobin by the percentage of red cells.

The figure of 5 millions as 100 per cent of red cells in colour and other index determinations is used for convenience in calculation and as Osgood (1926-27) says 'any other figure would do, if it were generally agreed upon'. Five millions is the generally accepted figure, but it must be clearly understood that it is not the average red cell count for either normal men or women.

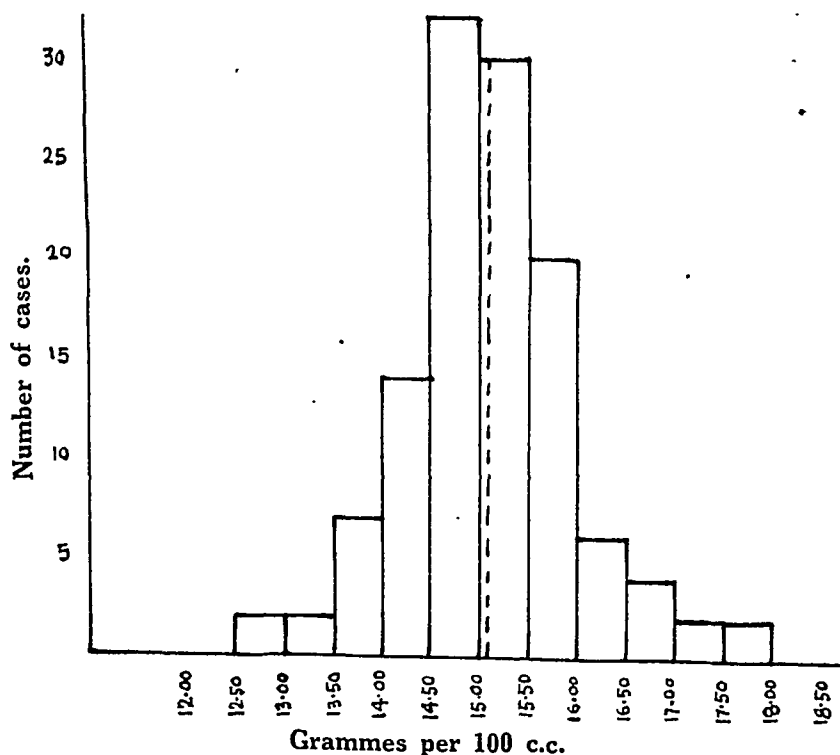


Fig. 3. Hæmoglobin coefficients in 121 men.

Our average hæmoglobin figure for 121 males being 15.37 g. and our average cell count for the same males being 5.11 millions, the hæmoglobin coefficient works out at 15.03 g. Fig. 3 is a histogram showing the frequency distribution of hæmoglobin coefficients. The figures range from 12.90 to 17.50. Mean (15.033 ± 0.054) and the median (15.058 ± 0.067) are very close. Standard deviation is 0.879 ± 0.038 and coefficient of variation is 5.819 ± 0.257 . The significant variation is 14.15 to 15.91; 75 per cent of our subjects fall within this range.

Our average colour index is 1.00 and the figures range from 0.86 to 1.17. Fig. 4 is a histogram giving the frequency distribution of our colour indices. The mean (1.002 ± 0.004) and median (1.006 ± 0.004) are very close. Standard deviation is 0.057 ± 0.003 and coefficient of variation is 5.637 ± 0.245 . Significant variation 0.95 to 1.06; 74 per cent of our subjects fall within this range.

Our hæmoglobin coefficient, 15.03, though it gives colour indices of 0.9 to 1.1 for 90 per cent of our subjects, is different from that of the other workers; Osgood (1926) 14.66; Wintrobe and Miller (*loc. cit.*) 13.66 (later Wintrobe, 1929, corrected this to 14.53); Haden (1933) 15.50; Napier and Das Gupta (1935, 1936) 13.78 for their first series of 50 subjects and 14.19 for the second series of 30 subjects giving an average of 13.94 for the 80 subjects.

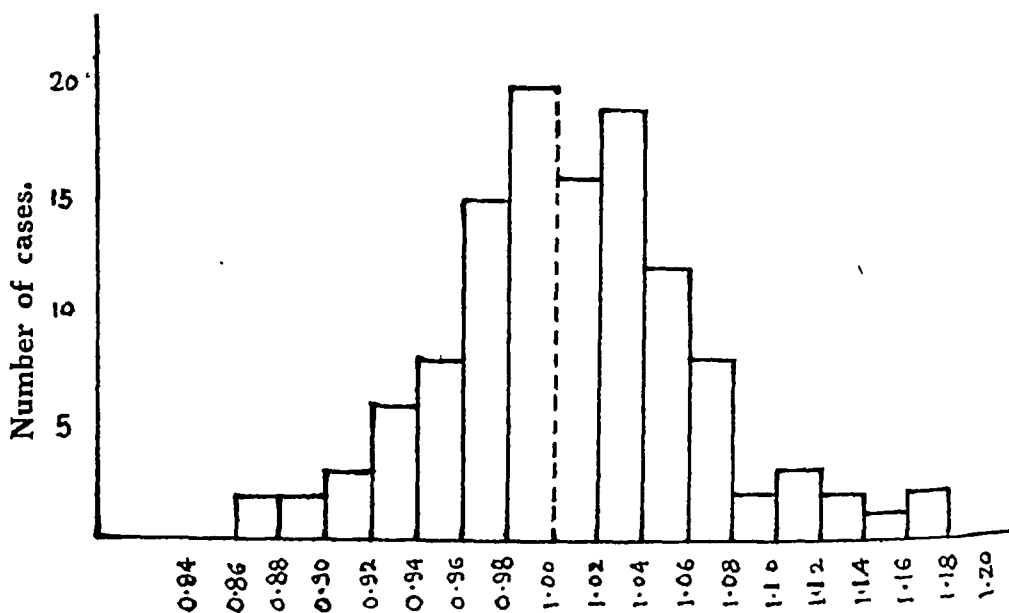


FIG. 4. Colour indices in 121 men.

Our hæmoglobin figure is strictly comparable only to the figures of Haden and Dill, but as Dill does not give his red cell counts, we must leave him out of the present discussion of hæmoglobin coefficient. Haden with an average hæmoglobin figure of 15.34 g. has a cell count of 4.95 millions, while we with a hæmoglobin figure of 15.37 g. have a cell count of 5.11 millions. These figures give hæmoglobin coefficients of 15.50 and 15.03 respectively. In view of the different red cell counts and hæmoglobin figures reported from different places by different workers, it does not seem that we are anywhere near having a common figure for normal hæmoglobin coefficient. It is not clear whether such a thing is possible. But the situation makes it imperative that further accurate studies should be made in as many different places as possible. In the meantime we commend Haden's (1933) suggestion that for each area the mean value for hæmoglobin and for the red cell count should be determined and those used for reporting to clinicians the hæmoglobin per cent and for calculating the colour and saturation indices.

TOTAL CELL VOLUME.

Our average figure for total cell volume is 41.72 c.c. per 100 c.c. of blood. Fig. 5 is the histogram showing the frequency distribution. The values range from 32.47 c.c. to 49.02 c.c., the mean and the median being 41.723 ± 0.183 and 41.674 ± 0.229 . The standard deviation is 2.978 ± 0.129 and the coefficient of variation is 7.132 ± 0.311 . Thus the significant variation is 38.75 to 44.70 and covers 72 per cent of our subjects.

Osgood (1926) obtained a mean value of 44.84 c.c. for 94 men; Wintrobe and Miller (*loc. cit.*) for 100 men 43.38 c.c.*; Napier and Das Gupta (1936) for 30 men 46.36 c.c.* The values obtained by these three workers are higher than that

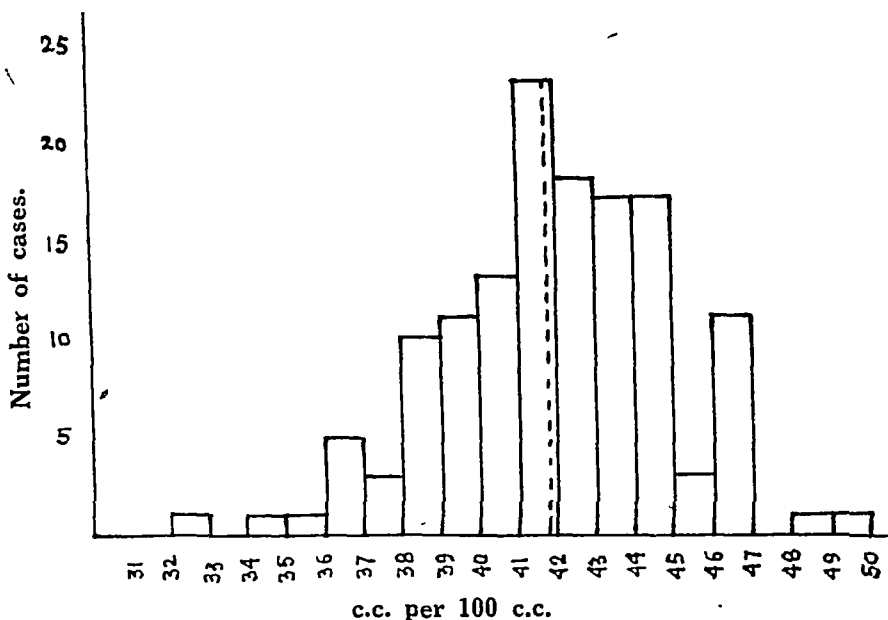


FIG. 5. Total cell volume in 121 men.

obtained by us, viz., 41.72 c.c. But it must be noted that the average red cell counts, Osgood's 5.49 millions, Wintrobe and Miller's 5.85 millions and Napier and Das Gupta's 5.53 millions are higher than our figure of 5.11 millions.

It is to be noted that our determinations were made on samples of bloods which were prevented from coagulating by the use of neutral potassium oxalate, 2 mg.

* Though Wintrobe and Miller used oxalated blood for determining their cell volume they do not give the figure they actually obtained, but instead they give a figure of 46.4 c.c. calculated by allowing for shrinkage of 6.7 per cent. We have re-converted their figure of 46.4 c.c. into the actual figure to make it comparable to our figure.

Similarly we have re-converted Napier and Das Gupta's figure of 50.53 c.c. to 46.36 c.c. by using their shrinkage factor of 1.09.

per c.c. of blood. This anticoagulant produces shrinkage of red cells, but we used it because an anticoagulant must be used and potassium oxalate is inexpensive and readily available in all laboratories and also because Osgood (1926) had used this anticoagulant and we were anxious to obtain figures comparable to his.

Osgood found the shrinkage to be 3.5 per cent and came to the conclusion that this is not large enough to alter the clinical significance of the volume index figures. But it will be noted that our figure is somewhat larger. If, however, the shrinkage is constant and the volume index calculation is based on standards determined by the potassium oxalate method the indices will be absolutely correct. When absolute figures for cell volume are needed, they could be obtained by the use of a factor calculated from the measurement of the extent of shrinkage caused by the use of potassium oxalate.

To determine shrinkage, oxalated blood is centrifuged against samples of the same blood to which isotonic anticoagulants, heparin or hirudin (1 mg. per 5 c.c.) has been added. It is assumed that neither heparin nor hirudin causes any contraction of the size of red cell. We give below (Table III) the results of determinations of shrinkage in ten different samples of blood using both hirudinized and heparinized controls. For these determinations we used the same speed and time (4,500 r.p.m. for 30 minutes) as in our own determination given in Table II.

TABLE III.

Effect of potassium oxalate on cell volume.

Number.	OXALATED.	HEPARINIZED.		HIRUDINIZED.	
	Cell volume, c.c. per 100 c.c.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.
1	38.35	40.75	5.89	40.61	5.57
2	39.71	41.99	5.44	41.95	5.34
3	40.35	42.61	5.30	42.71	5.53
4	45.77	48.58	5.78	48.69	5.99
5	48.99	51.81	5.44	51.89	5.59
6	40.91	43.47	5.89	43.42	5.78
7	40.43	43.06	6.11	42.87	5.70
8	40.57	43.17	6.02	43.02	5.69
9	46.48	49.33	5.79	49.29	5.71
10	41.15	43.95	6.37	43.88	6.22
AVERAGE ..	42.27	44.87	5.79	44.83	5.71

The values obtained by us show the shrinkage to be 5.79 per cent and 5.71 per cent of the volume of packed cells in heparinized blood and hirudinized blood respectively. The shrinkage is thus practically the same in both cases, the slight difference being within experimental error.

We should have thought that the amount of shrinkage obtained by different workers using the same anticoagulants would be the same under identical experimental conditions. But we are surprised to find it is not so. Osgood (1926) found the shrinkage to be 3.5 per cent, while we using the same technique obtained a figure of 5.75 per cent. We may state here that, while our figure is based on the examination of 10 samples, Osgood based his figure on only two samples. But we do not believe that this fact explains the difference, because none of our individual determinations give as low a figure as that of Osgood.

Still other figures are reported in literature. Haden (1929-30) gives a shrinkage value of 8.0 per cent and 8.24 per cent using 25 mg. of potassium oxalate and 20 mg. of potassium oxalate for 10 c.c. of blood respectively. Wintrobe (1929, 1931, 1932) gives three different figures at three different times, 3.7 per cent, 5.75 per cent, and 8.2 per cent, but he nowhere discusses the reasons for his obtaining such widely differing values. It is to be noted that his first figure tallies with that of Osgood and the second with ours and the third value with that of Haden. His first figure (3.7 per cent) is based on two determinations and the second (5.7 per cent) is based on 16 determinations; for these 16 determinations he does not give individual data. Later (Wintrobe, 1932) he gives still another figure (8.2 per cent) but does not give any data on which he bases this value. He does not discuss in his last paper (Wintrobe, 1932) how or why he obtained this very high figure. As far as we have been able to find he does not even state in his last paper that he had obtained lower figures before.

Osgood and Wintrobe used heparin and Haden used hirudin as the isotonic anticoagulant for their shrinkage determinations, but Napier and Das Gupta (1936) used both heparin and hirudin and with heparin they say they obtained for 7 cases a mean figure of 6.86 per cent and with hirudin in 10 cases a mean figure of 9.19 per cent. From the figures given in Table V of their paper it seems that the shrinkage value they obtained with heparin was 8.07 per cent and with hirudin 6.86 per cent. They do not give individual data for their determinations, but they proceed to say that 'if the 17 cases are taken together 7.94 per cent is the shrinkage'. We do not know how this figure is arrived at nor does it seem valid because an average is sought to be derived from too widely differing values. They further state that as their mean figure of 7.94 is very close to Wintrobe's figure of 8.2 per cent and as their numbers were small, they have accepted Wintrobe's figure. We have stated above that Wintrobe gives three different figures and though 8.2 per cent is the value he gives in his last paper (Wintrobe, 1932), he nowhere states the number of cases on which this figure is based.

Osgood writing as recently as 1935, still takes shrinkage due to oxalate to be 3.5 per cent of the volume of packed cells. The whole situation seemed to us to be highly confused. We have, therefore, made an attempt to study the question further. The factors involved seem to be the isotonic anticoagulants used, the quantity of the oxalate, and the speed and duration of centrifugation. Osgood and Wintrobe used heparin as the isotonic anticoagulant and Haden used hirudin, while Napier and Das Gupta used both heparin and hirudin. These later workers

obtained different values with those two isotonic anticoagulants. The position regarding the quantity of oxalate used is simple. All workers have used the customary amount of oxalate, i.e., 2 mg. of dry neutral potassium oxalate per c.c. of blood. We have adhered to this quantity. But different speeds of centrifugation were used by different workers. Osgood used a speed of 4,500 r.p.m. We also used the same speed. But Wintrobe used two speeds, first 3,500 r.p.m. and later 3,000 r.p.m., and Haden, and Napier and Das Gupta 2,500 r.p.m.

We have made parallel determinations on six bloods to determine the effect of isotonic anticoagulants heparin and hirudin, and the effect of speed. Results are given in Tables IV and V. It is to be noted that the duration of centrifugation necessary to get constant volume of cells varies with the speed. Centrifugation is continued until constant volume is attained. Time required to attain a constant volume was 30 minutes, 40 minutes, and 90 minutes at the speeds of 4,500, 3,500, and 2,500 r.p.m., respectively.

TABLE IV.

Cell volume and shrinkage percentages at different speeds.

Speed and time.	Number.	OXALATED.	HIRUDINIZED.		HEPARINIZED.	
		Cell volume, c.c. per 100 c.c.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.
4,500 r.p.m., 30 minutes.	5	48.99	51.89	5.59	51.81	5.44
	6	40.91	43.42	5.78	43.47	5.89
	7	40.43	42.87	5.70	43.06	6.11
	8	40.57	43.02	5.69	43.17	6.02
	9	46.48	49.29	5.71	49.33	5.79
	10	41.15	43.88	6.22	43.95	6.37
	AVERAGE	43.09	45.73	5.78	45.80	5.91
3,500 r.p.m., 40 minutes.	5	49.42	52.31	5.52	52.40	5.69
	6	41.30	43.82	5.75	43.79	5.71
	7	40.80	43.33	5.85	43.40	5.99
	8	40.69	43.20	5.81	43.23	5.88
	9	46.83	49.70	5.82	49.71	5.79
	10	41.35	44.00	6.02	44.13	6.30
	AVERAGE	43.40	46.06	5.80	46.11	5.88

TABLE IV—concl'd.

Speed and time.	Number.	OXALATED.	HIRUDINIZED.		HEPARINIZED.	
		Cell volume, c.c. per 100 c.c.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.
2,500 r.p.m., 90 minutes.	5	50.19	53.17	5.59	53.13	5.53
	6	41.91	44.42	5.67	44.47	5.76
	7	41.25	43.75	5.71	43.98	6.21
	8	41.20	43.67	5.66	43.85	6.00
	9	47.45	50.38	5.82	50.29	5.64
	10	42.47	45.24	6.12	45.26	6.16
	AVERAGE	44.08	46.77	5.76	46.83	5.87

TABLE V.

Average figures for cell volume and shrinkage percentage at different speeds.

Speed, r.p.m.	OXALATED.	HIRUDINIZED.		HEPARINIZED.	
	Cell volume, c.c. per 100 c.c.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.	Cell volume, c.c. per 100 c.c.	Shrinkage, per cent.
4,500	43.09	45.73	5.78	45.80	5.91
3,500	43.40	46.06	5.80	46.11	5.88
2,500	44.08	46.77	5.76	46.83	5.87
AVERAGE ..	43.52	46.19	5.78	46.28	5.89

All the figures given in Tables IV and V are the averages of duplicate determinations. The maximum variation between the duplicates was 1.9 per cent. It will thus be seen that the variation in the average volume of packed cells at different speeds of centrifugation is within the experimental error. It is also obvious that the isotonic anticoagulants used in the experiment do not give different values for shrinkage.

Our work does not explain how or why the different workers have obtained such widely varying values for shrinkage, nor is it clear to us how Napier and Das Gupta obtained different values with the isotonic anticoagulants, heparin and hirudin. It is obvious that further work on this point is necessary to clear the issue.

VOLUME COEFFICIENT AND VOLUME INDEX.

Just as the colour index expresses the ratio of the amount of hæmoglobin per red cell of the individual to that of the average normal, similarly volume index expresses the ratio of the volume of the red cell of the individual to that of the average normal. It is determined in the same way as the colour index with the

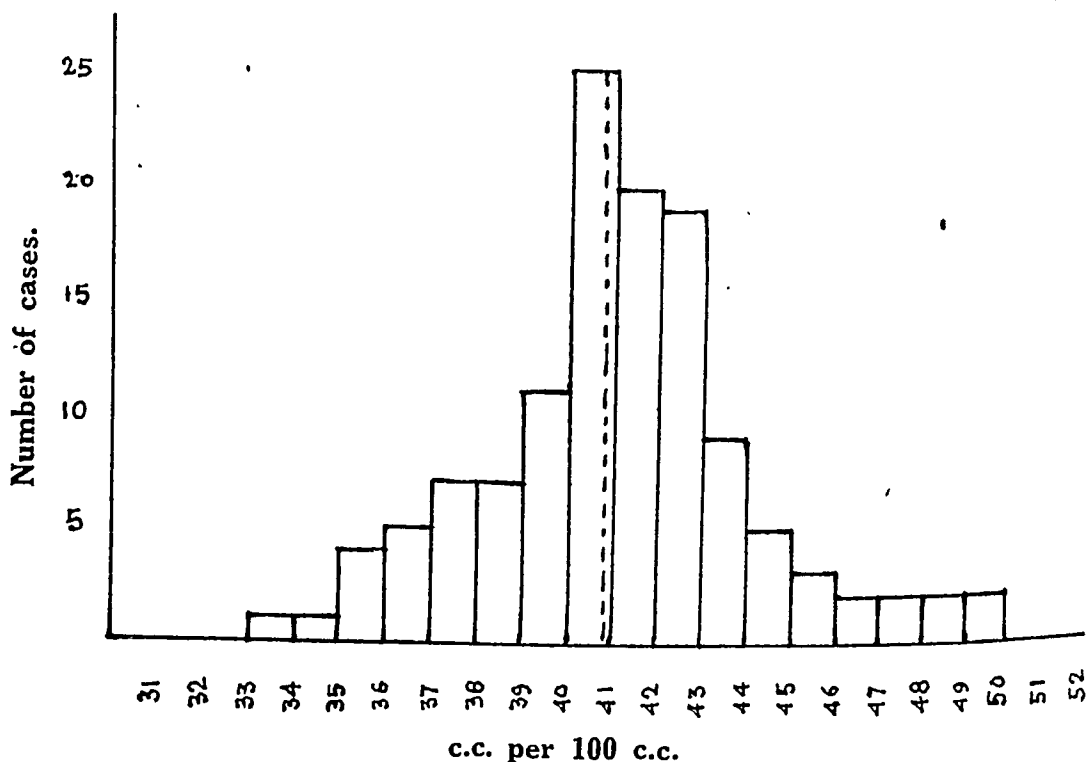


FIG. 6. Cell volume coefficients in 121 men.

difference that cell volume percentage is substituted for hæmoglobin percentage, i.e., the volume of the cells expressed as percentage of the average normal volume is divided by the percentage of red cells. The average volume of cells in 100 c.c. of blood of healthy persons of the same sex and age group calculated to red cell count of 5 millions is the normal standard, i.e., normal volume coefficient and is taken as 100 for the calculation of the percentage of red cell volume. For the determination of percentage of red cells the figure of 5 millions is taken as 100 per cent for reasons already given.

Our average figure for total cell volume for 121 males being 41.72 c.c. per 100 c.c. of blood and our average red cell count for the same subjects being 5.11

millions, our volume coefficient works out to be 40.83 c.c. Fig. 6 is a histogram showing the frequency distribution of the volume coefficients. The figures range from 33.71 c.c. to 49.93 c.c. The mean (40.83 ± 0.166) and the median (40.98 ± 0.209) are very close. The standard deviation is 2.723 ± 0.118 and the coefficient of variation is 6.654 ± 0.290 . Thus the significant variation is 38.11 to 43.55 and covers 75 per cent of our subjects.

Osgood (1926) gets a volume coefficient of 40.8 c.c., Wintrobe and Miller (1929) 37.08 c.c., Napier and Das Gupta (1936) 41.92 c.c. Our mean volume coefficient 40.83 c.c. compares very closely to that of Osgood.

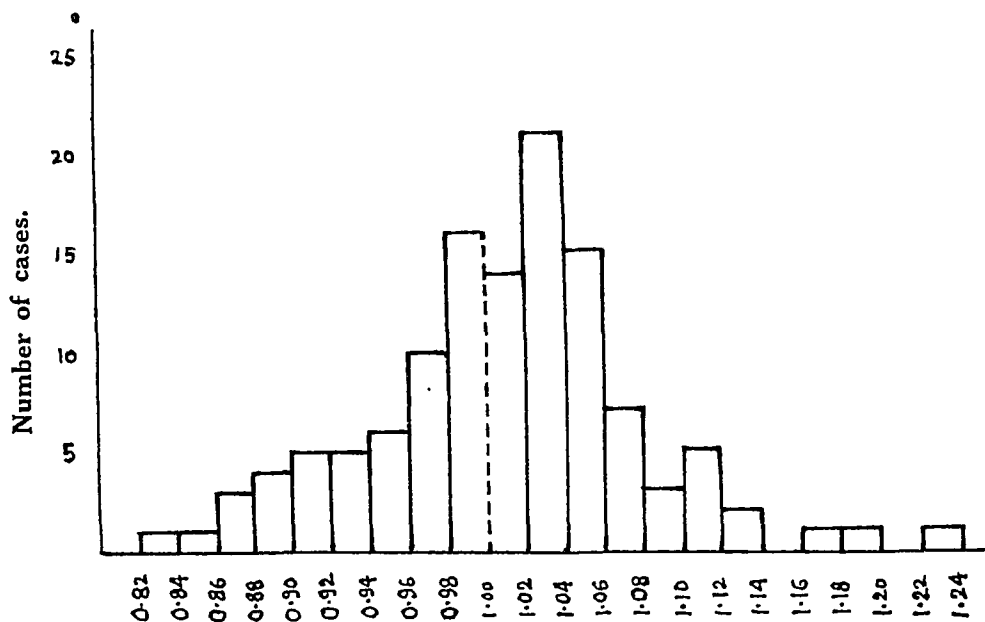


FIG. 7. Volume indices in 121 men.

Our average volume index is 1.00, the values ranging from 0.83 to 1.22. Fig. 7 is a histogram giving the frequency distribution of our volume indices. The mean (1.002 ± 0.004) and the median (1.014 ± 0.005) are close. Standard deviation is 0.068 ± 0.003 and coefficient of variation is 6.736 ± 0.293 . Significant variation is 0.93 to 1.07 covering 76 per cent of our subjects.

In the study of anæmias the size of red cell has proved of great diagnostic and prognostic value. Size has been expressed either as volume of the cell or its diameter. Capps (quoted by Osgood, 1926) showed the value of volume index in the study of anæmias, while Price-Jones (1933) introduced accurate methods for measuring the diameter of the cell. We do not propose to enter into the relative

merits of the two procedures here, except to say that Wintrobe (1924-25) states that 'the thickness of cells is as variable as the diameter or even more so'. Also the measurement of the diameter of red cells is a very laborious process and the error of the method is large while the accurate measurement of volume index is a comparatively simple procedure. We consider that the determination of the volume index gives all the information needed in clinical work.

SATURATION INDEX.

Haden (1923) pointed out that colour index is not an expression of the saturation of red cells with hæmoglobin, but merely expresses the relation of hæmoglobin to

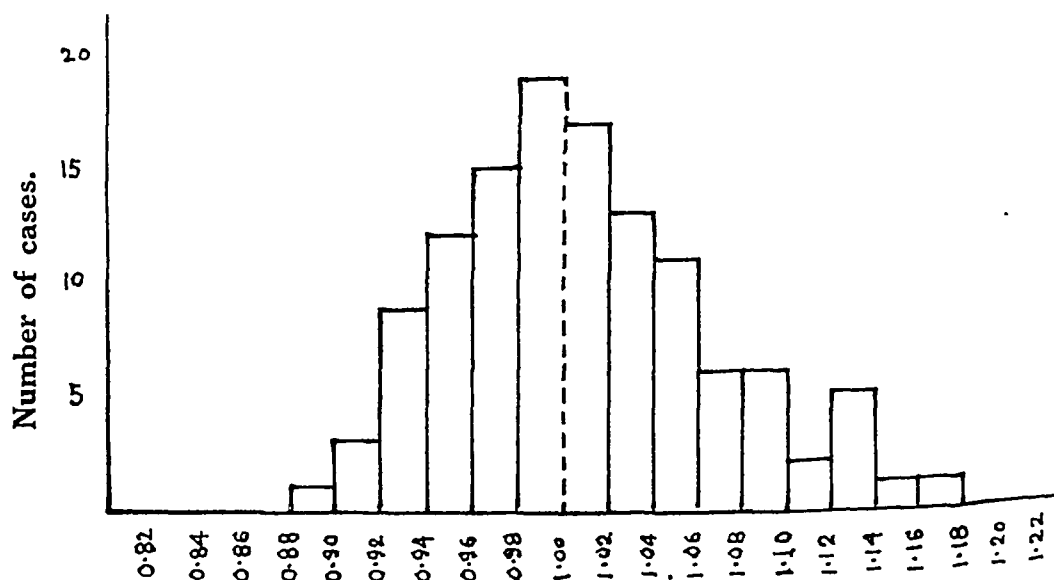


FIG. 8. Saturation indices in 121 men.

the number of red cells. To denote the amount of hæmoglobin per unit volume of cells he introduced the concept 'saturation index'. This is determined by dividing the colour index by the volume index. It is, however, to be noted that in normal cases when both the number and volume of cells are normal the colour index and the saturation index are synonymous.

Our average saturation index is 1.00, the figures ranging from 0.89 to 1.16. Fig. 8 is the histogram giving the frequency distribution of our saturation indices. The mean 1.002 ± 0.004 and the median 1.002 ± 0.004 are close. Standard deviation is 0.057 ± 0.003 and coefficient of variation is 5.649 ± 0.245 . Significant variation is thus 0.95 to 1.06 and covers 72 per cent of our subjects.

SUMMARY.

1. Standards for the normal averages have been worked out by the study of bloods of 121 healthy young men from the Bombay Presidency between the ages 19 and 30 years. The findings are tabulated below :—

Averages and ranges of variation in normal findings. 121 men.

	Mean.	Minimum.	Maximum.	Standard deviation.	Percentage of subjects within significant variation.
Red cells, millions per c.mm.	5.11	4.07	5.95	± 0.38	71
Hæmoglobin, grammes per 100 c.c.	15.37	12.75	17.69	± 0.96	72
Cell volume, c.c. per 100 c.c.	41.72	32.47	49.02	± 2.98	72
Hæmoglobin coefficient, grammes per 100 c.c.	15.03	12.90	17.50	± 0.88	75
Volume coefficient, c.c. per 100 c.c.	40.83	33.71	49.93	± 2.72	75
Colour index ..	1.00	0.86	1.17	± 0.057	74
Volume index ..	1.00	0.83	1.22	± 0.068	76
Saturation index ..	1.00	0.89	1.16	± 0.057	72

2. The cell volume given in the table is the average of actual volumes determined in oxalated blood. The question of shrinkage of cells in oxalated blood is discussed at some length and necessity of further work on the subject indicated.

3. Particular attention is drawn to our hæmoglobin figure. All the determinations on which it is based were made with the van Slyke oxygen capacity method. It is pointed out that though our average hæmoglobin figure varies widely from other normal average given in literature, it agrees with the normal averages of the only two other large series reported in literature (Haden), in which hæmoglobin was determined by van Slyke's method. Need of further work on the subject is stressed.

4. It is pointed out that though our average hæmoglobin figure is comparable to and agrees with the averages obtained by Haden, our hæmoglobin coefficient does not agree even with his, because of the difference in the red cell count figures. The unsatisfactory position regarding a common normal hæmoglobin coefficient is discussed and further work suggested.

5. An alternative colorimetric method to van Slyke's oxygen capacity method for hæmoglobin determination in clinical work is recommended.

Our thanks are due to Dr. Jivaraj Mehta, the Dean, Dr. V. R. Khanolkar, the Professor of Pathology, of the S. G. S. Medical College, for giving us facilities for obtaining samples of blood and to the students who volunteered.

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HÆMATOLOGICAL STUDIES IN INDIANS.

Part VIII.

ANALYSIS OF THE HÆMATOLOGICAL FINDINGS IN 52 CASES OF ANÆMIA AMONGST PREGNANT TEA-GARDEN COOLIE WOMEN.

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THIS investigation was commenced at the beginning of October 1935. The population from which the patients were taken was the same as in the previous investigations already reported (Parts VI and VII of this series). The 'normal' non-pregnant women were taken from amongst coolies who were attending for the systematic inspections after the obviously anæmic patients had been excluded, and the 'normal' pregnant women were taken from the 'pregnant parades' referred to in Part VII of this series, again after the obviously anæmic patients had been excluded.

The anæmic patients were selected, because they had symptoms of anæmia, because they were obviously anæmic, or because the Tallqvist scale gave a reading below 50 per cent*.

The conditions were the same as those in the previous investigations and the same methods were followed, except that the centrifuge used before had burnt out and an inferior one had to be used. This did not attain the same speed, and, although a longer time was allowed to compensate for this, the packing was not so complete and a factor—calculated subsequently—to compensate for this has been introduced; the figures for cell volume and the figures calculated therefrom cannot be accepted as absolutely correct though we believe that the error is very slight.

* It will be seen that when some of these were investigated the hæmoglobin estimated by more accurate methods was actually higher than some of those in the 'normal' group.

'NORMAL' NON-PREGNANT FEMALE COOLIES.

The findings in 20 non-pregnant women are summarized in Table I:—

TABLE I.

	Maximum.	Minimum.	Mean.	Standard deviation.
Hæmoglobin in grammes per 100 c.c. blood ..	13·60	9·20	10·80	± 2·30
Red blood cells in millions	5·49	3·57	4·55	± 0·65
Cell volume per cent	42·00	30·00	34·60	± 3·10
Mean corpuscular volume	88·70	64·10	77·30	± 7·70
Mean corpuscular hæmoglobin	30·00	18·80	24·50	± 3·00
Mean corpuscular hæmoglobin concentration ..	35·50	29·30	31·20	± 1·70

The mean hæmoglobin in this series falls half-way between that of the females in the two previous 'normal' groups reported in Parts III and V of this series. The other figures are materially the same.

'NORMAL' PREGNANT WOMEN.

The findings in 40 'normal' pregnant women are summarized in Table II:—

TABLE II.

	Maximum.	Minimum.	Mean.	Standard deviation.
Hæmoglobin in grammes per 100 c.c. blood ..	12·51	9·62	10·70	± 1·60
Red blood cells in millions	5·96	3·68	4·65	± 0·62
Cell volume per cent	37·80	29·00	32·80	± 2·30
Mean corpuscular volume	90·20	59·40	72·10	± 8·10
Mean corpuscular hæmoglobin	29·50	18·20	23·80	± 2·90
Mean corpuscular hæmoglobin concentration ..	39·20	27·80	32·60	± 1·80
Reticulocytes	4·70	0·40	2·10	± 1·80

There is little difference, and certainly no significant difference, between the values we obtained in the two groups, pregnant and non-pregnant. This confirms our previous observation (Part VII) that in pregnancy there is no general lowering of the hæmoglobin level.

ANÆMIC PREGNANT WOMEN.

There were in this series 52 cases.

Clinical symptoms.—Very often the patients made no complaint of ill health until pressed and then they admitted becoming easily tired and suffering from some shortness of breath. In others, these symptoms prevented them from working. Soreness of the mouth or tongue was seldom admitted.

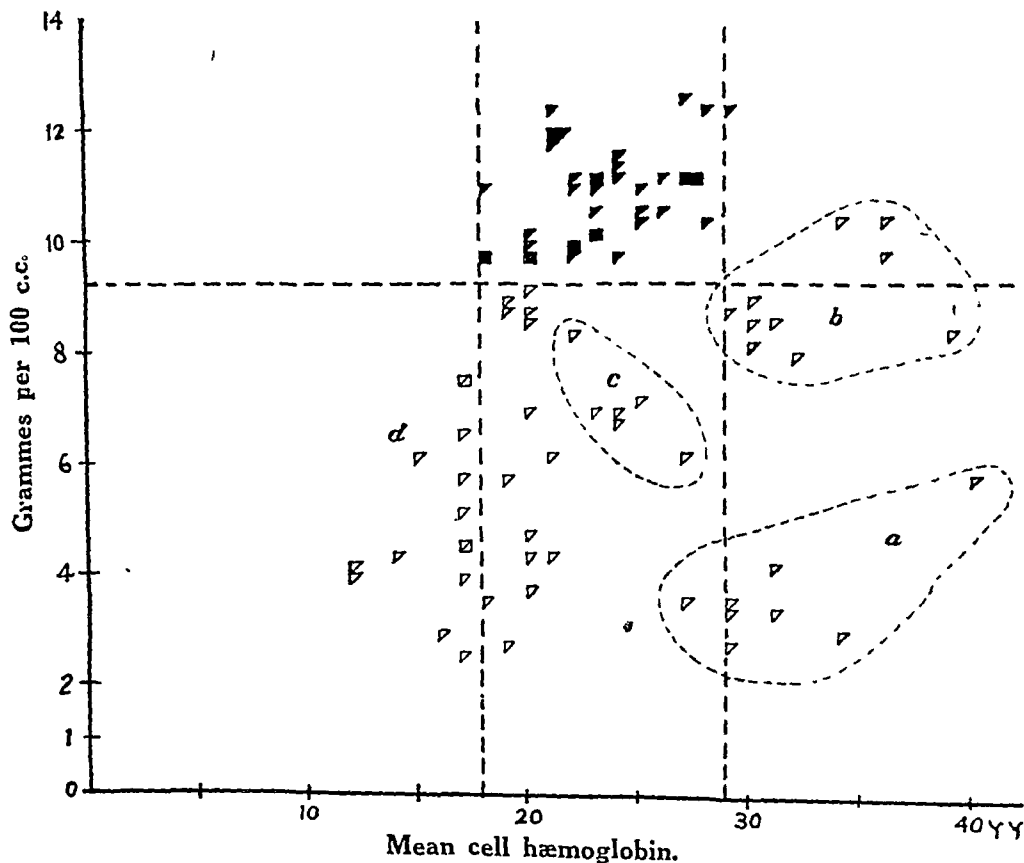
Their general appearance usually was pale, and the tongue had the wash-leather appearance but was seldom red or sore. Some œdema of the feet was very common, and also puffiness of the face. The skin was often lustreless and dry. Splenic enlargement was not uncommon and is referred to again.

Koilonychia was not noted in any case, nor were any eye changes suggestive of vitamin-A deficiency.

The distribution of these cases according to the degree of anæmia and the mean corpuscular hæmoglobin is best shown in the Chart in which also the 40 pregnant non-anæmic cases are shown:—

CHART.

Showing distribution of 40 'normal' and 52 anæmic pregnant females according to their hæmoglobin percentages and mean corpuscular hæmoglobin.



It will be seen that the anæmic cases fall into four more-or-less distinct groups, and that the non-anæmic coolies form a fifth distinct group.

The four groups are as follows:—

	Cases.
(a) Hyperchromic — marked anæmia (2.75 g. to 5.77 g.)	8
(b) " — slight anæmia (7.97 g. to 10.31 g.)	10
(c) Orthochromic — moderate anæmia (6.18 g. to 8.25 g.)	6
(d) Hypochromic — a continuous group with all degrees (less than of anæmia (2.47 g. to 9.07 g.)	28
MCH 22γ)	
Total ..	52

The reason why we have grouped these cases according to the degree of hæmoglobin content of the cells (i.e., the MCH) rather than according to their size (i.e., the MCV) is that, as we explained above, there is some uncertainty regarding the cell-volume estimations.

Were they arranged according to the size of the cell (MCV), very little rearrangement would be necessary:—

in group (b), No. 27 is microcytic,
in group (c), No. 48 is very definitely microcytic,
and in group (d) Nos. 11 and 31 are normocytic.

The justification for our arbitrary grouping.—In order to justify this arbitrary grouping we have analysed certain hæmatological and biochemical findings in each group separately.

Hæmoglobin concentration.—The hæmoglobin concentration in the various groups is interesting. In group (d) there is a close correlation between the degree of anæmia and the mean corpuscular hæmoglobin concentration percentage (MCHC), but in all except three cases the MCHC is *below* 30.0. In the other three groups the same relationship is noticed, but in all except one case the MCHC is *above* 30.0.

If we take an arbitrary figure of 8.5 grammes of hæmoglobin and exclude all cases above this we find that in every case of hypochromic anæmia the MCHC is *below* 30.0 and of the rest in all except one case it is *above* 30.0.

The hypochromic group is again clearly differentiated from the rest by its low corpuscular hæmoglobin concentration.

Reticulocytes.—The counts when the patients were first seen can be grouped as follows:—

TABLE III.

	(a).	(b).	(c).	(d).	TOTAL.
0 to 1	1	..	1
1 to 2 ..	2	1	3
2 to 3 ..	2	1	1	3	7

TABLE III—concl'd.

	(a).	(b).	(c).	(d).	TOTAL.
3 to 4	2	2
4 to 5 ..	1	3	1	6	11
5 to 6 ..	1	1	..	1	3
6 to 7	1	..	5	6
7 to 8 ..	2	1	1	4	8
8 to 9	1	1	1	3
9 to 10	1	..	1	2
10 to 15	1	3	4
15 or over	2	2

The mean reticulocyte count of the whole series was 5.75 per cent. The various groups did not show any significant differences though the counts in group (a) were the lowest.

The leucocyte count.—There was little difference between the means in the different groups. They were : (a) 8,360, (b) 9,010, (c) 8,050, and (d) 9,370 per c.mm. The difference of one thousand between (a) and (d) is far below the significance level.

Differential nucleated-cell count.—This was done in only 27 instances : therefore some of the groups are very small and consequently are not worth careful analysis.

Nucleated red cells.—Nucleated red cells were observed in 17 cases ; the number varied from 1 to 11 nucleated red cells in a count of 200 nucleated cells. The relative incidence of nucleated red cells in each of the four different groups was about the same.

Eosinophils.—In group (a) in four cases the count was recorded—the mean of the four was 2.87 per cent, in six cases of group (b) the mean was 5.21 per cent, and the mean of the remaining 16 cases was 7.06 per cent. These figures are all lower than we obtained with normal females of the same class of population.

Neutrophils.—The mean count in groups (a) and (b) was 51 per cent against 57 per cent in group (d) : the difference is not significant.

Van den Bergh.—The 'direct' test was in every case negative. The 'indirect' test gave the results shown in Table IV. An accurate quantitative estimation was not done on account of the difficulties, already referred to in a previous paper, in obtaining a standard that would match. We have recently overcome this

difficulty and we are now satisfied that the provisional values we gave are approximately correct.

TABLE IV.

Results.	Approximate concentration of bilirubin in mg. per 100.	'Normal' pregnant women.	INCIDENCE IN DIFFERENT ANÆMIA GROUPS.			
			(a).	(b).	(c).	(d).
Negative	Less than 0.5	33	..	2	2	12
(+)	0.5 to 1.0	3	1	3
+	1.0 to 2.0	4	5	6	3	7
++	2.0 to 3.0	..	2	1	..	4
+++	over 3.0	..	1	1	..	1

The van den Bergh in No. 27 was +, and in Nos. 11, 31, and 48 was negative, so that rearranging according to cell volume rather than hæmoglobin content would mean the transfer of one + from (a) to (d) and one negative from (d) to (c).

There is a very marked difference between the van den Bergh in the hyperchromic, (a) and (b), and in the hypochromic, (d), groups. Statistically, the difference in the incidence of a positive van den Bergh is very significant*—43.05 per cent, i.e., about three times the standard deviation (14.76 per cent).

The difference between the van den Bergh in the 'normal' pregnant women and those of the hypochromic group is also very significant (difference 44.44 per cent, standard deviation = ± 10.6 per cent).

The rearrangement of this group according to the volume of the cells diminishes this difference only very slightly and leaves it still very significant.

Gastric analyses.—These proved very unpopular amongst the coolies and, in order not to jeopardize the whole inquiry by estranging the labour force, they were discontinued early in the inquiry. In three cases an adequate number of samples was taken: in all three free acid was found. Two of these cases belonged to group (d) and one to group (a).

Summary.—We can now give a short summary of the main features of the four groups:—

(a) A severe anæmia, macrocytic and hyperchromic, with a mean corpuscular hæmoglobin concentration above 30 per cent, a van den Bergh (indirect) positive and free acid in the gastric juice (one case only tested in this series but the observation has been confirmed in another series).

(b) A lesser degree of anæmia with the other features similar but again less pronounced.

* As in previous papers in this series, whenever the word 'significant' is used, statistical significance of a 0.05 order is implied.

- (c) A hypochromic microcytic anæmia of all degrees of severity, with a low mean corpuscular hæmoglobin concentration (below 30 per cent), with an indirect van den Bergh which is usually negative but sometimes positive, and free acid in the gastric juice.
- (d) An orthochromic normocytic anæmia of moderate severity, with a mean corpuscular hæmoglobin concentration above 30 per cent, and an indirect van den Bergh that is sometimes positive. This is probably not a distinct group.

Ætiological factors.—It seems very probable that the ætiological factors involved in the production of the anæmia in these different groups are not the same and therefore it seems worth analysing some of the ætiological data collected, separately for each group, as we did in the case of the hæmatological data.

Age.—The age grouping in the four anæmic and the non-anæmic groups is given in Table V :—

TABLE V.

Ages.	(a).	(b).	(c).	(d).	Non-anæmic.
15 to 19	2	1	..	5	4
20 to 24	3	2	1	11	12
25 to 29	3	3	2	6	11
30 to 34	..	4	2	4	8
35 to 39	1	2	5

Group (a) is certainly the youngest group, but there is nothing in the age distribution to which significance can be attached.

Previous pregnancies.—Arranged according to the number of previous pregnancies the cases are grouped as given in Table VI :—

TABLE VI.

Para.	(a).	(b).	(c).	(d).	Total.	Non-anæmic.
I ..	4	1	1	6	12	9
II ..	2	4	2	11	19	4
III ..	1	2	..	3	6	9
IV ..	1	..	2	2	5	5
V	2	..	3	5	4
VI or more	..	1	1	3	5	5

There appears to be a distinct tendency for the severe macrocytic anæmias to occur in the early pregnancies, but the difference in the distribution even between groups (a) and (d) is not significant statistically. Another point of interest is the very high incidence of anæmia in the second pregnancy, particularly so in the hypochromic group.

TABLE VII.

Month of pregnancy.	(a).	(b).	(c).	(d).	Non-anæmic.
2nd	1	..
3rd	1	..
4th	1	1
5th	1	..	2	1
6th ..	1	6	3
		2			5
7th ..	2	3	..	4	11
8th ..	4	5	5	9	16
9th ..	1	1	1	4	4
		16		17	31

There is evidence of some difference in the groups here. If we group together the cases examined in the third trimester and those examined in the first two trimesters separately, for the microcytic group (d) and the macrocytic groups (a) and (b), we find that the difference is only just below the significance level (difference 0.382, standard deviation = ± 0.146). If the microcytic cases are compared with the rest, the difference is definitely 'significant'.

Diet.—By casual inquiry we obtained very little information of value and it is proposed to carry out a general dietary survey when opportunity arises. The caloric value of the adult diet we calculated at about 2,200 calories. The story given was that they took meat once a week, usually mutton, chicken, or duck; about half the patients took fish also once a week; very few took milk or eggs. The daily food consisted of rice, dal, and vegetables.

There is so little difference in the dietary histories given and their accuracy is so doubtful that, despite an impression that diet is an important factor, we do not consider that it would be profitable to analyse the records.

Malarial infection and splenic enlargement.—Malarial parasites were found in a few cases but the gardens are situated in an intensely malarious area and the actual presence of parasites at the time of examination is a matter of chance. A better indication of the extent to which the patients have suffered from malaria in the past is the size of their spleens.

The cases with and without splenic enlargement arranged according to the four anæmic groups and 40 pregnant controls* are as follows :—

	Enlarged.	Not below the costal margin.	Per cent enlarged.
(a) ..	4	4	33·3
(b) ..	2	8	..
(c)	6	0
(d) ..	4	24	14·3
Non-anæmics	2	32	5·9

The difference between the percentage of enlarged spleens in the hyperchromic groups (a) and (b) and in the hypochromic group (d) is just short of the significance level (standard deviation = ± 0.106). The exclusion of the group (b), the slightly anæmic group, would make the difference significant, so also would the inclusion of the orthochromic group (c) amongst the hypochromic cases. Nos. 27, 11, 31, and 48 show no splenic enlargement so that rearrangement according to cell volume would make little difference.

Helminthic infections.—Egg counts were done on samples of the stools of all the patients and pregnant controls; the findings can be summarized as follows :—

	Anæmics, per cent.	Non-anæmics, per cent.
Hookworm infection in ..	96	82
Whipworm „ „ ..	94	90
Round-worm „ „ ..	80	85
Triple „ „ ..	76	68

* In four cases the notes were incomplete.

The round-worm infections were very high, up to 60,000 eggs per gramme, but were quite definitely higher in the non-anæmic women. The whipworm egg counts were about the same in each group.

Though the hookworm egg incidence (82 per cent) was high in the non-anæmic cases, the counts were definitely higher in the anæmic groups and these counts have been examined separately for each of the anæmic groups, and compared with one another and with those of the non-anæmic patients.

TABLE VIII.

Hookworm infection.

Eggs per gramme of stool.	ANÆMIC PATIENTS OF VARIOUS GROUPS.				Non-anæmic.
	(a).	(b).	(c).	(d).	
30,000 +	1	..
10,000 to 19,900	3	1
5,000 to 9,900 ..	2	1 3	1	8	2
2,000 to 4,900 ..	1	6	1	7	8
Less than 2,000 ..	5	3	3	9	29
Not recorded	1

We have usually accepted 5,000 eggs per gramme as being the pathogenic level, though of course the figure is an arbitrary one. In the incidence of this degree of infection, there is a sharp contrast between the hyperchromic and the hypochromic groups, the incidence in the latter being much higher. The difference between the incidence of a heavy infection (5,000 eggs or more per gramme) in the two groups is not quite significant (difference = 0.262, standard deviation = ± 0.141). The difference between the hyperchromic and the non-anæmic groups is far below the significance level, but the difference between the non-anæmic and the hypochromic groups is very significant.

These figures suggest that hookworm infection is an important factor in producing the anæmia in the hypochromic cases, but that in the hyperchromic group it is less important as the heavy-infection incidence is not much greater than in the non-anæmic series.

We will consider this from another point of view. The correlation coefficients of the hæmoglobin content of the blood and the hookworm egg count in the 18 hyperchromic cases and in the 28 hypochromic cases have been calculated, using

$$\text{the formula } r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

In the hyperchromic series $r = +0.0111$, an entirely negligible figure, and in the hypochromic group $r = -0.4776$, which with the standard error at ± 0.1924 is distinctly 'significant'.

The correlationship is shown quite well in Table IX in which the hypochromic cases are grouped according to their hæmoglobin percentages and egg counts:—

TABLE IX.

Hæmoglobin, grammes per 100 c.c.	HOOKWORM EGGS PER GRAMME OF STOOL.			
	10,000 +	5,000 +	2,000 +	Below 2,000.
9 grammes +	1
8 „ less than 9	3	1
7 „ „ „ 8	1	1
6 „ „ „ 7	2	..	2
5 „ „ „ 6 ..	1	2	..	3
4 „ „ „ 5 ..	1	1	1	1
3 „ „ „ 4 ..	1	2	1	..
2 „ „ „ 3 ..	1	1	1	..

Subsequent progress.—In this investigation we did not follow the cases systematically, and a subsequent record of the blood condition, or a history of death having occurred, was only obtained in 23 cases.

Two patients had died at the time of or soon after delivery. The second blood examination was done after intervals of one to three months, subsequent to delivery in most cases. In most cases iron in large doses had been prescribed, but had not been taken regularly by the majority of the women.

The progress and the nature of the blood picture at the second examination in these 21 cases are shown in Table X:—

TABLE X.

Subsequent progress.

Difference between first and final examination in grammes per 100 c.c. of hæmoglobin.	GROUP AT FIRST EXAMINATION.				
	(a).	(b).	(c).	(d).	
Decreased	2	..	3	
Constant or increased but less than 1 g.	1	..	5	
Increased more than 1 g. „ „ „ 2 g.	1	..	1	
„ „ „ 2 g. „ „ „ 3 g.	2	
„ „ „ 3 g. „ „ „ 4 g.	2	1	
„ „ „ 4 g. „ „ „ 5 g.	1	
„ „ „ 5 g. „ „ „ 6 g. ..	1	
„ „ „ 6 g. „ „ „ 10 g. ..	1	
Nature of blood picture at second examination.	{ Microcytic .. Normocytic .. Macrocytic ..	1	1	..	12
		..	2	2	1
		1	1
Died ..	1	..	1	..	

Both of the patients in group (a) had been delivered for three months at the time of the second examination; in one the blood picture had returned to the normal microcytic picture of the coolie class, the other had retained the macrocytic tendency, but this was less marked.

Of the cases in group (b), in one, three months after delivery, the blood picture had returned to the normal of the coolie class, in one the hæmoglobin had remained constant at about the normal level, but the size of the cells was reduced (MCV = 85); and in two in which the second count had been taken soon after delivery there was a marked decrease in hæmoglobin and in one a decrease in the size of the

cell. In the two cases in group (c) there was little change in the size of the cells, and in group (d) all but one maintained the microcytic nature of the blood picture: in this case subsequent to delivery there was considerable increase in hæmoglobin and the MCV had increased to 97.

To summarize, it can be said that there was little change in the size of red cells except in some of those cases in which there was a marked increase or decrease in the hæmoglobin; the decrease in hæmoglobin is in most instances dependent on loss of blood at delivery, as in the two cases in group (b). Of the six macrocytic cases examined a second time only two retained their macrocytic nature.

DISCUSSION ON ÆTIOLOGICAL FACTORS.

Little assistance is obtained from the grouping, according to age, and, though in the table showing the grouping according to previous pregnancies there appears to be a distinct tendency for group (a) cases to occur in the early pregnancies, this conclusion will not bear statistical analysis.

On the other hand there is a significant difference in the microcytic group and the two macrocytic groups in relation to the month of pregnancy.

If the anæmia were entirely independent of the pregnancy one would expect that the cases would be distributed evenly amongst the nine months of pregnancy, but one has to remember that few of these coolies were sufficiently ill to report to the dispensary and that the majority only came under observation because they applied for 'pregnancy leave' in the last trimester. On the other hand, if the anæmia were due mainly to the pregnancy one would expect the maximum effect during the last trimester.

In groups (a) and (b), and also (c), the anæmia does not seem to have been sufficiently pronounced in the early stages of pregnancy to bring the coolies to the dispensary for medical attention before they would normally report, but in group (d) there is a more even distribution amongst the months and there is evidence of some factor which may cause severe anæmia in the early months of pregnancy and which is therefore probably independent of the pregnancy itself.

The figures showing the distribution of splenic enlargement amongst the various groups are very suggestive. If the hyperchromic cases with severe anæmia are compared with the hypochromic cases the difference is significant, but the numbers involved are very small, and if the whole hyperchromic group is included the difference is only just below the significance level. The incidence of splenic enlargement in the hypochromic group is little more than that of the general adult population.

Malaria may be considered the only important cause of splenic enlargement in this district, as kala-azar is almost unknown, and this disease, together with certain other diseases that might cause splenic enlargement, was excluded in the cases concerned.

We may conclude that the evidence does point to malaria and/or splenic enlargement as playing some part in the ætiology of the hyperchromic type of anæmia in these pregnant women.

The effect of hookworm infection seems very clear. In the hypochromic group the infections are very much heavier than in the controls or the other groups.

The incidence of a pathogenic infection is significantly higher in the hypochromic group than in the controls, and in this group the coefficient of correlation between the degree of anæmia and the hookworm egg counts is significant.

Therefore, there is strong evidence of hookworm infection being an ætiological factor in this type of anæmia. On the other hand, there is complete lack of evidence of the association between the hyperchromic anæmia and hookworm infection.

In a previous paper (*vide* Part VI) we were able to show that the anæmia in the general population in this district was almost all microcytic hypochromic anæmia easily cured by iron administration. We have found that the incidence of anæmia in different gardens in this district varied from 6 to 12 per cent. The anæmia amongst pregnant women was 15·8 per cent (*vide* Part VII). We must not attempt to deduce too much from the percentages just quoted but they do seem to us to suggest very strongly that the hypochromic anæmia which constitutes a little over half of the anæmia in this series is the same hypochromic anæmia as that occurring in the general population and that the higher rate of anæmia amongst pregnant women can be accounted for by the superadded hyperchromic anæmia.

This hypochromic microcytic anæmia conforms in every way with the hypochromic microcytic anæmia we found in the general population, except that in this series the response to treatment was not so good as when we ourselves superintended systematic treatment with large doses of iron.

To summarize our findings, regarding the severe hyperchromic anæmia, group (a), there is a tendency for a higher incidence in the first pregnancy and in the later months of pregnancy and there is evidence of an association with splenic enlargement but none with hookworm infection.

The less severe hyperchromic anæmia also shows a tendency to higher incidence in the later months of pregnancy, shows a lesser degree of association with splenic enlargement, but also shows no association with hookworm infection. Little can be said about group (c) except that there is no evidence of any association with hookworm infection or splenic enlargement.

CONCLUSION.

In conclusion, one may say that the anæmia in these pregnant women is of two main types; one is a hypochromic microcytic anæmia caused mainly by hookworm infection though possibly exaggerated by other factors including pregnancy, the other is a hyperchromic macrocytic anæmia in which the pregnancy itself appears to be an important causative factor and which is associated with splenic enlargement but not with hookworm infection.

There is also a group of cases in which the hæmoglobin is almost within the normal range but in which the size of the cells is large; this type is apparently ætiologically similar to the severe type of macrocytic anæmia and is associated with the pregnancy, but it may be looked upon as an almost physiological anæmia of pregnancy. Finally, there is a small group of cases of a normocytic anæmia of a moderate degree of severity which differs from each of the other two types in some particulars and which is possibly a combination of the two types.

PROTOCOL.

Summary of hæmatological data of 52 cases of *anæmia in pregnant codie women.*

Serial number.	Age.	Month of pregnancy.	Para.	Hæmoglobin in g. per 100 c.c.	MCV.	MCH.	MOHC.	Lymphocytes percentage.	Van den Bergh indirect.	Hookworm egg count in 1,000 eggs per gramme.	Splenic enlargement below costal margin in inches.	Group.
1	22	6	II	7.56	64.0	17.7	27.8	?	—	1.80	n	(d)
2	18	4	II	2.88	71.7	16.9	23.6	39.0	—	6.00	p	(d)
3	16	3	II	2.47	82.5	17.2	20.9	20.5	+	36.20	p	(d)
4	22	8	I	3.43	91.5	29.0	31.8	37.0	++	8.60	4"	(a)
5	35	8	VI	3.85	55.0	12.4	22.6	70.0	—	2.40	n	(d)
6	20	2	V	4.26	66.2	14.8	22.4	34.5	—	0.40	n	(d)
7	23	5	III	5.08	65.3	17.4	26.7	23.0	±	1.20	n	(d)
8	30	5	VI	8.25	118.8	39.8	33.6	30.0	+	0.60	3"	(b)
9	35	9	VII	6.73	76.6	24.3	30.9	25.0	—	0.00	n	(c)
10	30	6	VII	7.56	65.6	17.7	27.0	..	—	2.40	2"	(d)
11	32	8	V	3.71	89.0	20.3	22.9	23.5	—	6.60	n	(d)
12	20	8	I	5.63	66.4	17.9	27.0	32.0	++	9.60	n	(d)
13	22	8	I	6.18	85.9	27.2	31.7	30.5	+	1.20	n	(c)
14	25	6	II	2.75	78.6	19.6	25.0	18.5	+	3.60	n	(d)
15	19	5	II	4.67	71.5	20.4	29.0	27.5	++	13.20	n	(d)

PROTOCOL—concl'd.

Serial number.	Age.	Month of pregnancy.	Para.	Hæmoglobin in g. per 100 c.c.	MCV.	MCH.	MCHC.	Lymphocytes percentage.	Van den Bergh indirect.	Hookworm egg count in 1,000 eggs per gramme.	Splenic enlargement below costal margin in inches.	Group.
16	38	8	VII	6.87	69.9	20.8	29.9	34.5	—	7.80	n	(d)
17	25	7	II	2.75	117.0	29.2	25.0	29.0	+	2.20	p	(a)
18	22	8	IV	5.08	55.0	13.2	24.2	35.0	+	5.40	n	(d)
19	25	8	III	10.31	92.7	34.1	36.8	63.5	+	3.00	2"	(b)
20	20	8	I	3.71	92.9	29.2	31.4	61.0	+	0.20	2"	(a)
21	17	7	I	8.52	93.3	30.0	32.0	34.5	—	9.40	n	(b)
22	29	7	II	10.31	105.6	34.4	36.3	34.5	++	0.00	n	(b)
23	22	6	I	3.85	65.0	17.2	26.5	..	—	19.60	n	(d)
24	25	7	II	3.57	67.3	18.4	27.4	62.0	++	9.00	n	(d)
25	25	9	I	5.77	70.4	19.8	28.1	55.5	+	1.80	n	(d)
26	16	7	I	6.18	58.4	15.8	27.1	76.0	±	5.80	n	(d)
27	20	8	II	8.11	75.3	30.8	40.4	44.5	+	4.40	n	(b)
28	25	8	III	9.76	105.2	36.0	34.2	31.5	+++	3.20	n	(b)
29	25	6	II	4.26	74.0	21.7	29.3	..	+++	8.60	n	(d)
30	30	7	V	7.97	92.7	32.1	34.5	19.5	—	2.20	n	(b)
31	16	7	I	4.26	91.7	20.5	22.4	30.0	—	2.40	n	(d)
32	32	8	II	7.15	82.7	25.7	31.1	..	+	4.80	n	(c)

33	18	7	I	2.88	106.0	34.6	32.7	..	+	1.20	n	(a)
34	24	8	III	3.71	91.1	27.6	30.4	..	++	1.20	n	(a)
35	26	8	II	6.87	77.1	24.6	31.9	..	±	6.00	n	(c)
36	27	6	II	5.77	116.5	41.5	35.6	..	+	0.20	p	(a)
37	33	9	III	8.66	69.7	20.6	29.7	..	—	3.20	n	(d)
38	30	8	V	6.46	62.6	17.7	28.3	..	+	1.20	2"	(d)
39	24	9	IV	5.50	61.1	17.6	27.5	..	—	12.00	n	(d)
40	22	8	II	8.80	66.3	19.7	29.8	..	±	3.20	n	(d)
41	22	9	I	5.50	63.9	17.7	27.8	..	+	0.80	n	(d)
42	28	8	III	9.07	64.6	20.2	31.3	..	—	0.80	n	(d)
43	16	9	I	4.12	106.4	33.2	31.2	..	+	0.00	n	(a)
44	22	7	II	8.52	65.5	20.5	31.3	..	+	2.00	n	(d)
45	26	6	II	8.66	59.9	18.5	30.9	0.60	n	(d)
46	30	8	IV	6.87	74.1	23.3	31.5	..	+	5.40	n	(c)
47	26	8	IV	3.57	108.2	32.7	30.2	..	++	6.20	n	(a)
48	25	8	IV	8.25	68.8	22.5	32.7	..	—	0.20	n	(c)
49	30	8	II	8.52	93.4	31.2	33.4	..	+	2.40	n	(b)
50	20	8	II	6.18	72.8	21.2	29.1	..	++	0.60	n	(d)
51	30	8	V	8.66	97.6	29.3	30.1	..	+	0.20	n	(b)
52	20	9	II	8.80	94.7	30.8	32.6	..	+	4.60	n	(b)

SOME OBSERVATIONS ON THE EXPERIMENTAL PRODUCTION OF CATARACT.

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THE problem of cataract deserves a certain amount of attention in India where its incidence is said to be high in certain regions, notably in the Punjab. The possibility of a dietary factor being involved was suggested by the work of Day and Langston (Day, Langston and O'Brien, 1931 ; Langston and Day, 1933 ; Day and Langston, 1934 ; Day, 1934), who succeeded in producing cataract in 92 per cent of rats fed on a vitamin-B₂ free diet. Later workers (Bourne and Pyke, 1935), however, with an exactly similar diet obtained an incidence of only 31 per cent. Mitchell and Dodge (1935), on the other hand, produced cataract in rats on a diet rich in lactose but without any vitamin deficiency, and maintained that some abnormality in carbohydrate metabolism was responsible for the condition. Day (1936) stated, however, that cataract following the feeding of lactose or galactose was different from that observed on a vitamin-B₂ deficient diet. Several workers have repeated Day and Langston's experiments but have never observed an incidence of cataract greater than about 30 per cent. The few observations recorded here were an attempt to elucidate the discrepancy and, if possible, to find out if some other factors were involved. This goal has not been achieved. The main object of publication, apart from certain other features which appear, is to guide others who may be tempted to try the modifications of diet which we have employed. The original observations of Day and Langston were made with the Sherman and Spohn (1923) diet vitamin B₁ being supplied in the form of an alcoholic extract of rice polishings evaporated on a little starch and mixed with the diet. Our modifications in the diet were made chiefly from the point of view that discrepancies in the results might be due to (i) the source of vitamin B₁ in the diet, and (ii) the nature of the starch fed. We previously made the observation while doing some work on the vitamin-B₁ assay of foods that two out of three rats fed with cobs of corn as a supplement to vitamin-B₁ free diet developed cataract. This led us to try the

addition of fresh green corn to the diet. The following diets were employed in the experiments recorded here :—

GROUP I. Sherman and Spohn diet ; an alcoholic extract of rice polishings equivalent to 10 per cent of polishings being incorporated in the diet as a source of vitamin B₁.

GROUP II. Similar diet to group I, but the rice-polishings extract was fed separately instead of being incorporated in the diet.

GROUP III. A vitamin-B₂ free diet with dextrin taking the place of maize starch and vitamin B₁ fed separately as a Kinnersley and Peters' extract from yeast.

GROUP IV. A similar diet to group III, except that green cobs of corn were fed as a source of vitamin B₁.

GROUP V. A low protein diet in which maize meal replaced most of the casein and also formed the source of vitamin B₁.

GROUP VI. Sherman and Spohn diet with the modification that cobs of corn were the source of B₁ factor.

GROUP VII. A diet containing 40 per cent lactose. Vitamin-B₁ extract from rice polishings was fed separately.

As these diets were either completely or partially deficient in vitamin B₂, the following signs of deficiency were looked for :—

- (i) Dermatitis, hyperæmia, and ulceration of the paws associated with the B₆ factor of the vitamin-B₂ group.
- (ii) Alopecia, falling out of hair associated with flavin deficiency in rats.
- (iii) Cataract, said to be due to vitamin-B₂ deficiency, or a disturbance of carbohydrate metabolism.
- (iv) Loss of weight and early death.

The results of these experiments are summarized in the Table :—

TABLE.

Group.	Diet.	Total number of rats.	Disease.	Number of rats affected.	Time of development of symptoms (days).	Incidence, per cent.
I.	<i>Sherman and Spohn diet.</i>					
	Casein 18	12	Cataract Dermatitis Alopecia	3 0 0	30, 32, 42	25 0 0
	Maize starch . . . 68					
	Ghee 8					
	Cod-liver oil . . . 2					
	Salt mixture . . . 4					
	B ₁ as rice-polishings extract mixed with the diet.					

TABLE—*contd.*

Group.	Diet.	Total number of rats.	Disease.	Number of rats affected.	Time of development of symptoms (days).	Incidence, per cent.
II.	<i>Sherman and Spohn diet.</i>					
	Same as above. B ₁ extract from rice polishings fed by the mouth separately.	20	Cataract	0	..	0
			Dermatitis	2	35, 37	10
			Alopecia	0	..	0
III.	<i>Vitamin-B₂ free diet.</i>					
	Casein 15	11	Cataract	1	45	9
	Dextrin 71					
	Agar agar 2					
	Ghee 5					
	Salt mixture 5					
	Cod-liver oil 2					
	Vitamin B ₁ as Peters' yeast extract.					
			Dermatitis	2	87, 83	18
			Alopecia	5	Irregular	45
IV.	Similar diet as (III). Cobs of corn formed the source of vitamin B ₁ .	6	Cataract	0	..	0
			Dermatitis	0	0	0
			Alopecia	0	..	0
V.	<i>Low protein maize diet.</i>					
	Maize meal 35	8	Cataract	1	46	12
	Maize starch 50					
	Ghee 8					
	Salt mixture 5					
	Cod-liver oil 2					
			Dermatitis	0	..	0
			Alopecia	2	91, 83	25
VI.	<i>Sherman and Spohn diet.</i>					
	Same as (I).	6	Cataract	0	..	0
	Vitamin B ₁ supplied as cobs of corn.					
			Dermatitis	0	..	0
			Alopecia	1	42	16

TABLE—concl'd.

Group.	Diet.	Total number of rats.	Disease.	Number of rats affected.	Time of development of symptoms (days).	Incidence, per cent.
VII.	<i>Lactose diet.</i>					
	Casein 18	6	Cataract Dermatitis Alopecia	0 0 0	0 0 0
	Maize starch . . . 38					
	Lactose 40					
	Ghee 8					
	Salt mixture . . . 4					
	Cod-liver oil . . . 2					
	Vitamin-B ₁ extract from rice polishings fed separately.					

A glance at the Table shows that the incidence of cataract, dermatitis, and alopecia varies considerably in the different groups. The maximum incidence of cataract is only 25 per cent. Several points, however, should be noted. In groups I and II, the only difference between the diets is the method of giving the alcoholic extract of rice polishings. In group II it is fed by the mouth, and in somewhat smaller quantity than would have been consumed in group I (0.25 c.c.; later increased to 0.5 c.c. per diem). In group I ten out of the twelve rats survived for thirty weeks and three developed cataract but no dermatitis or alopecia was observed. Yeast and marmite were fed to those affected but no improvement in the eye lens was noted. This group showed the greatest incidence of cataract.

In group II, two out of the twenty rats developed dermatitis which was promptly cured by feeding vitamin B₆ prepared from whey from which the flavin had been removed. The remaining eighteen rats survived only for five weeks without showing any signs of cataract or other skin conditions. Groups III and IV were fed the usual vitamin-B₂ free diet which is employed in this laboratory for routine tests. In this diet dextrin replaced maize starch and vitamin B₁ was supplied as extract of yeast in group III, and as 1 gramme of green corn in group IV. In group III only one of the eleven animals developed cataract, but two developed dermatitis and five alopecia. The presence of both dermatitis and alopecia was observed in some of those but no animal with cataract showed any skin symptoms. The dermatitis and alopecia were readily cured by flavin and vitamin-B₆ preparations from whey of milk. The rats, which showed no signs of deficiency, survived for periods of 4 to 21 weeks.

In group IV, no signs of any deficiency were noted in the six rats during a period of 15 weeks when the experiment was discontinued. Apparently the cobs of corn had supplied all the missing factors. Group V was fed the low proteins diet containing 35 per cent maize meal as the only source of vitamin B₁ and protein. Only one rat out of eight developed cataract in forty-six days. In this particular case, the cataract improved after feeding a rice-polishings extract for about thirty days and the animal survived for 14 weeks. Two rats from this group developed alopecia which was also cured by feeding rice-polishings extract. The other animals all died within six weeks. In group VI where 1 gramme of fresh cobs of corn was fed as the source of vitamin B₁ with a Sherman and Spohn diet, only one rat showed a mild alopecia. All the animals survived for 15 weeks when the experiment was discontinued. In group VII, lactose diet with rice polish extract, no case of cataract or dermatitis was observed in the nine-week period of the experiment.

The results obtained from these experiments would appear to show that a pure vitamin-B₂ deficiency *per se* is not a cause of cataract. It is possible that, along with some unknown factor, vitamin B₂ may be involved although the fact that dermatitis and cataract were not observed together in the same animal would tend to discountenance this theory. A factor tending to complicate the problem is the time of appearance of cataract, which might suggest that it is possibly a symptom of animals which are capable of surviving an absolute or relative deficiency of vitamin B₂. It is also possible that in a series of rats which have survived a certain period of time, individual differences in their constitutional make-up may determine the appearance of cataract or skin symptoms.

It is significant to note that in our experiments, the appearance of cataract, in those animals in which it developed, was much earlier than has been observed by other workers. The animals which did not get the disease in 4½ to 6½ weeks did not get it at all although some of them survived as long as 231 days (group I). This observation supports the view that only certain individuals, which are more susceptible, develop the condition.

We desire to express our thanks to Professor H. Ellis C. Wilson for his help and advice in carrying out this investigation.

SUMMARY.

1. Cataract appears to be associated with diets which are deficient, among other things, in vitamins of the B₂ group.
2. The evidence does not favour the hypothesis that vitamin B₆ (the anti-dermatitis factor) or flavin is the cause of the condition.
3. The results would appear to show that possibly only certain susceptible members of a group of animals may develop the condition.

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ENDEMIC FLUOROSIS IN THE MADRAS PRESIDENCY.

BY

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INTRODUCTION.

THE occurrence of this condition in the Madras Presidency has been recorded in a preliminary note by Shortt, Pandit and Raghavachari (1937). The present account is a more complete description of the condition based on a careful investigation of ten cases from the affected area which were admitted into the Presidency General Hospital, Madras, for this specific purpose.

The cases were chiefly in an advanced stage of the 'disease' and the description, therefore, applies more especially to individuals who have been subjected for periods of forty years or more to the influence of drinking waters containing comparatively large quantities of fluorine compounds. At the same time, opportunity was taken, in the preliminary field investigation, to study the earlier manifestations of the condition and especially the effect produced on the teeth of children, giving rise to the condition widely known as 'mottled enamel'.

Our reasons for publishing our findings in considerable detail are two in number :—

The first of these was that a search of the literature has failed to reveal any previous account of this intoxication comparable to conditions prevailing in the area under consideration, either in the extent of country affected or in the severity of the manifestations. The reason for the latter probably lies in the fact that the majority of the references, with some notable exceptions, relate to the United States of America where, as soon as the fluorine causation of the early stages of the intoxication, as evidenced by mottling of the enamel of children, was established

in any area, steps were taken to change or otherwise deal with the water supply. In this way, the later pathological manifestations associated with very prolonged use of waters highly charged with fluorine were avoided and only the early changes of mottled enamel were noted and described. In the affected areas of the Madras Presidency, on the other hand, as the disease has not hitherto been traced to its real cause, no changes in the water supply have been made apart from action taken, completely empirically, by the villagers themselves in moving their villages in order to obtain a 'better' water supply since Indian villagers attribute, with and without reason, many of their ills to their water supplies. In an area so widely affected some of these changes must almost certainly have been for the worse. The 'disease' therefore, since it comes in the first instance from the soil, might truly be described as being in this area 'as old as the hills'.

The second reason for our account is that, so far as we are aware, there has been no previous record of this condition existing in any part of India and we hope that this description of it in Madras may bring to light any other affected areas, if they exist, and so lead to action which will ameliorate the condition of the inhabitants who add to their disabilities with every drink of water they take.

A short study of a large number of cases in the field and the more detailed study of the ten cases admitted to hospital bring to light the fact that there is an extraordinary degree of uniformity in signs and symptoms shown by all cases in any particular stage to which the intoxication has advanced. This uniformity makes it unnecessary, even in a first detailed account of the condition in India, to describe the symptoms and clinical findings in each case individually. In giving an account of the clinical and radiological findings, therefore, we have contented ourselves with a general description which will be found to apply to most cases and from which the individual variations will be small and unimportant. This method of treatment has not been quite so easily applicable in the case of the biochemical examinations made on these patients and here the details in the individual cases have, in addition, been tabulated. In applying this method of presenting our findings we commence with a systematic clinical description of the cases followed by a description of the bony changes, as shown by radiology, which account for many of the clinical manifestations. This is followed by an account of the biochemical investigations.

CLINICAL FINDINGS.

General clinical picture.

This has been given, very shortly, in the communication cited above but, for the sake of completeness, may be repeated here, with some additions; before the more detailed clinical findings are entered into.

In children born and brought up in the area under consideration, there is a very high incidence of 'mottled enamel' in the teeth. This mottling is especially characteristic of the permanent teeth but may also be seen in the deciduous teeth in areas where the fluorine content of the drinking water is especially high. As the teeth are presumably influenced in the process of formation, the fact that deciduous teeth show the defect proves that a very prolonged exposure to fluorine containing waters is not necessary, provided the fluorine content be high.

The usual course of events in the permanent teeth appears to be somewhat as follows :—

The enamel of the teeth loses its glistening appearance and becomes a dead white like chalk. This change is not evidenced uniformly over the whole area of the tooth but tends to take one of three forms :—

- (a) a banding of the tooth by a horizontal stratum of chalky whiteness with, sometimes, subsidiary but less well-developed strata.
- (b) a more or less centrally placed area of whiteness surrounded by a more normal appearance of the tooth.
- (c) an irregular pattern of patches of whiteness.

After this whiteness of the teeth has lasted some time and if the consumption of water from the same source continues, the whiteness is replaced by chocolate-coloured markings occupying exactly the same areas as were previously white. All these appearances are best shown by the central upper incisors but any or all of the teeth may be affected in either jaw and the appearance is characteristic and most striking. Apart from the colour defects noted, the teeth of the children do not appear to be any better or worse than those of children in unaffected areas. Hutchinsonian teeth appear to be common, but whether due to fluorine intoxication or to syphilis is uncertain. The children, apart from the dental condition noted, do not appear to suffer in any way from the intake of fluorine and there would appear to be an interval, extending from childhood to about 25 to 30 years of age, during which few or no ill-effects are exhibited. Whether this is because the maturing body is able in some way to deal with the excess of fluorine or, as is more probable, the effects of the latter are cumulative, we have not yet had the opportunity to investigate. At about 30 years of age, however, the first symptoms of intoxication appear. This is evidenced by a recurrent general tingling sensation, in the limbs or over the body in general. Pain and stiffness next appear, especially in the lumbar region of the spine but also involving the dorsal and cervical regions.

The stiffness increases until the entire spine, including the cervical region, appears to be one continuous column of bone, producing a condition of ' poker back '. Such patients to turn the head must turn the whole body, in fact the spine loses its flexibility almost entirely; accompanying the spinal disability, there is stiffness of various joints due to infiltration by bony material of the periarticular tissues, tendinous insertions of muscles and interosseous fasciæ. This leads to various other disabilities such as loss of the power of squatting. The bony skeleton of the thorax is markedly affected and the ribs become rigidly fixed at their junctions with the spine. This results in their complete inability to allow expansion of the cavity of the thorax and breathing becomes entirely abdominal, while the chest assumes a barrel-shaped outline flattened anteriorly. By the time this condition is reached, the individual is between 30 and 40 years of age and the later and final stages of the intoxication are imminent.

The patients exhibit a definite cachexia; there is loss of appetite and general emaciation. Symptoms of pressure on the spinal cord may appear, due, as will be seen later, to bony encroachment on the spinal canal. There is loss of sphincter control in the latest stages and impotence is common. The patient is finally completely bed-ridden, while the mental powers are unimpaired. Death usually occurs due to some intercurrent disease.

The picture here drawn may be said to be the typical march of events but there are minor variations in individual cases and all in a village do not exhibit the intoxication to the same degree. This, however, is what might be expected as the water drunk while at work in distant fields might not be that of the village of residence and so, all the inhabitants of a village are not subjected to uniform conditions.

Special clinical findings.

These will be considered as briefly as possible under certain heads and the account given should be understood to be a summary of the conditions found in the ten cases investigated. We have not considered it necessary to go in great detail into the findings in respect of each physiological system because, on the whole, the clinical examination of the cases yielded no striking features. Thus, the chief clinical symptoms were the physical disabilities present due to the mechanical effects of bony growths around the spinal column and joints, and in tendinous insertions and interosseous ligaments.

Résumé of systematic clinical findings.

History of illness.—The first symptoms noticed are tingling sensations all over the body with pains in the limbs. The duration varied between one and ten years, the average period since commencement of symptoms being four years. In all cases, there had been a gradual deterioration in condition with lapse of time until the stage described in an earlier part of this communication had been reached.

Present general condition.—The patients were undernourished in most cases and with limitation of movement in various joints; in some cases bed-ridden.

Teeth.—These were in poor condition generally, discoloured, and with many missing. Pyorrhœa alveolaris was present in most cases. The discoloration was largely due to excessive use of tobacco which is grown in the district.

Cardio-vascular system.—In most cases the heart boundaries and sounds were normal but the majority showed some tachycardia, possibly temporary and due to the excitement of examination.

Respiratory system.—Percussion and auscultation revealed nothing abnormal. The respiration rate varied from 18 to 44 and averaged 26. The vital capacity varied greatly with individuals, 1,100 c.c. to 2,375 c.c. The chest was almost immobile due to fixation of the ribs and breathing in some cases was purely abdominal. The chest, seen from in front, was flattened anteriorly, with the lateral margins curved to give a barrel-shaped outline. The intercostal spaces were sunken owing to atrophy of the unused muscles.

Osseous system.—There was synostosis of the entire vertebral column, usually commencing in the lumbar region, while the cervical region was the last to be seriously affected. In some of the cases, the vertebral column was completely rigid and neither antero-posterior nor lateral movement was possible. Kyphosis or scoliosis was present in some cases. The costo-chondral junctions appeared to be ossified. There were bony outgrowths at the tendinous insertions of important muscles into the long bones. The edges of the ribs, especially the lower borders, showed irregularities due to bony outgrowths. The femora in some cases were bowed laterally.

Joints, ligaments, and tendons.—Limitation of movement and pain in joints were present in all cases. Hip, knee, shoulder, elbow, and ankle joints were especially affected. The lower part of the tendo achilles was ossified in several cases.

Muscular system.—Generalized wasting of all the muscles was present in all cases, sometimes accompanied by pain on pressure. Part of this, as in the case of the intercostal muscles, may have been a disuse atrophy.

Nervous system.—Mental condition and speech in all the cases were unaffected and the cranial nerves normal. The tone of the muscles was poor. The knee-jerks in most of the cases were exaggerated. The plantar reflex was flexor except in the two most advanced cases in which it was extensor. The abdominal reflexes were unimpaired or even brisk except in these two cases, in which they were lost. These cases also showed marked ankle clonus.

There was some diminution in pain and thermal sensation in several cases. Thermal sensation was lost over the lower extremities in the two most severe cases. Tactile and vibration sense was also lost over the same area and in these two cases there was also loss of sphincter control.

Endocrines.—Nothing abnormal was noted.

Sexual functions.—These were normal in the earlier stages, impaired or completely lost in the later.

Ophthalmoscopic examination.—No characteristic changes were noted.

Special investigations.—*Blood.* Bleeding time and coagulation time revealed nothing of note. There was a slight degree of anæmia in most of the cases as revealed by blood counts and hæmoglobin percentage. Differential counts were not distinctive although some cases showed a high eosinophile count. *Fæces.* Three of the cases showed ova of ascaris on a single examination.

RADIOLOGICAL FINDINGS.

General radiological picture.

Nearly the whole skeleton of each of the ten patients under observation was radiographed. For purposes of description the cases may be graded into three radiological groups based on the progression of bony changes which was found to correspond closely with the clinical severity and duration of the disease. Among the cases examined, there were no representatives of the early stages of intoxication and it is probable that these would yield little radiological evidence.

The first and earliest cases of the ten examined formed a group showing changes in the cancellous tissue of the bones, especially those of the trunk, viz., spine, pelvis, and ribs. These changes took the form of increased density of bone with exaggeration of the trabeculation and diminution of the medullary area. The bones affected in order of frequency were spine, pelvis, ribs, os calcis, ends of long bones, shafts of fibula, tibia, radius, ulna, femur, metacarpals, and carpals.

The second group shows a great exaggeration of the changes noted above. The density of cancellous bone is greatly increased and the trabeculation is still more pronounced. The outlines of bones, such as the vertebræ and pelvis, stand out clear-cut and dense and the spinal ligaments show early calcification changes. The

*Group III—late cases.**Bones of trunk.*

(a) *Spine*.—The interspinal ligaments, especially the anterior, are uniformly calcified.

The vertebræ appear very dense and osteophytic outgrowths from pedicles and laminae are present in all regions of the spine.

In one case, in the dorso-lumbar region, there is diffuse bony infiltration into the lumen of the spinal canal. There is complete bridging over by bone of the spaces occupied by the intervertebral discs in the mid-dorsal and dorso-lumbar regions so as to make the spine a rigid bony column. This is accompanied by some rotation of the vertebræ.

(b) *Ribs*.—These show marked osteophytic growths, chiefly directed downwards from the lower margins, simulating the effect of dependent icicles.

(c) *Pelvis*.—The pubic, trochanteric and acetabular areas, the crest of the ileum and the bony pelvis all show extreme density and osteophytic outgrowths. The sacro-iliac joints are completely synchondrosed.

In some cases, the lower outlet to the pelvis appears to be almost completely bridged by osteophytic outgrowths.

Long bones.

These show the changes already described in the preceding groups but in an enhanced degree, the osteophytic growths and calcification being definitely related to ligaments, tendinous insertions of muscles, and interosseous membranes.

Other bones.

The remarks made with regard to the long bones apply equally here.

Skull.—The skull in no case throughout the series shows any definite or characteristic changes.

BIOCHEMICAL INVESTIGATIONS.

These were limited to examinations of the blood and urine. While it cannot be said that the investigation yielded any results of special interest, with the possible exception that fluorine in unusual concentration was demonstrated in the urine, yet the records of such examinations in chronic fluorine intoxication are so few in the literature that we have considered it advisable to give the results in full detail. The biochemical investigations were handicapped as regards investigation of the amounts of fluorine in blood and urine by the want of any well-established and proven methods of estimation and we, therefore, give our findings with some reserve until more exact quantitative methods are devised.

The various estimations carried out and the methods employed are noted below while the detailed results are given in the tables which follow.

Blood.

Blood sugar.—This was estimated by a modified Folin's method. The figure fell within normal limits in six cases, above in two and below in two. The average figure was within normal limits.

PLATE XIII.

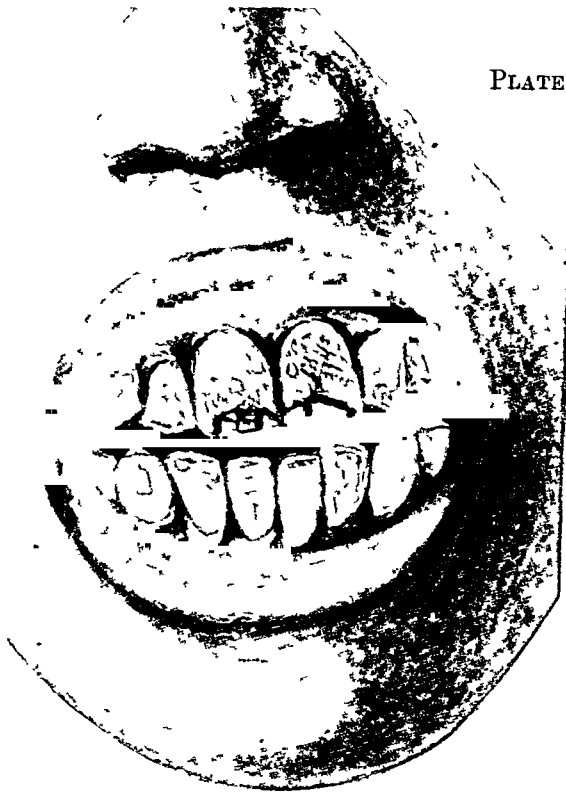


Fig. 1

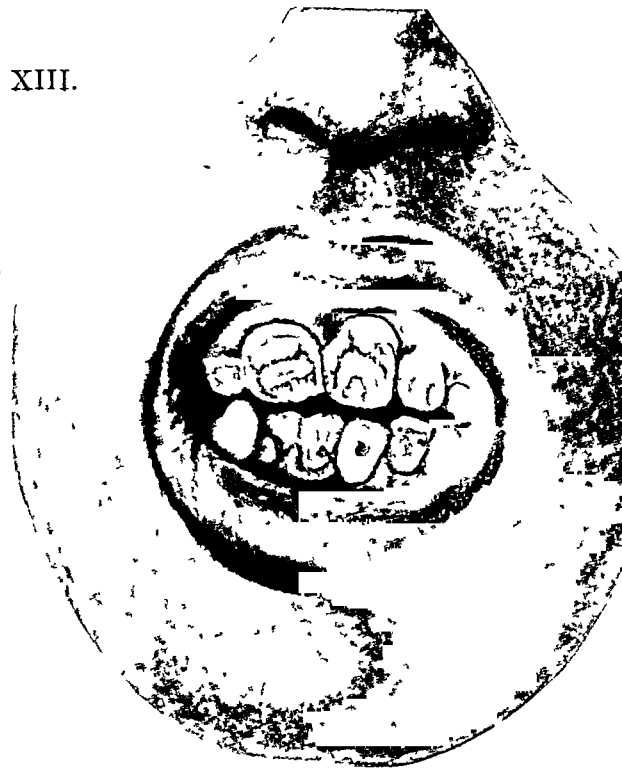


Fig. 2.

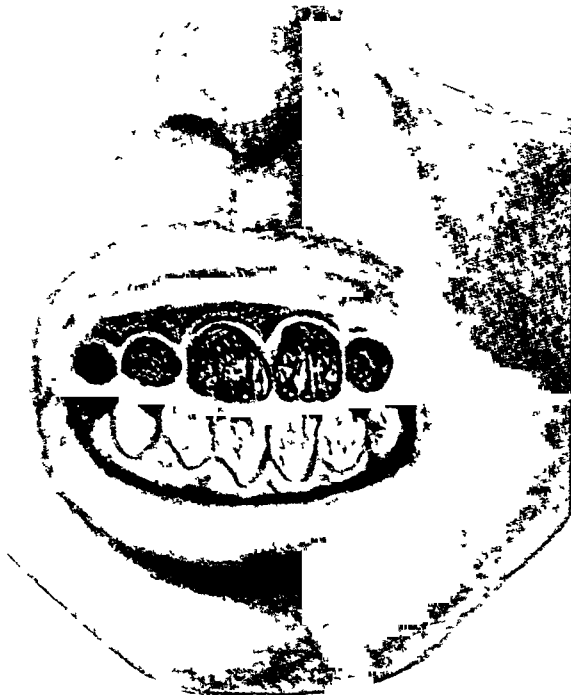


Fig. 3.



Fig. 4.

Condition shown in teeth of children in area of endemic fluorosis. Figs. 1, 2, and 3 show irregular mottling and Fig. 4 banded mottling. Figs 3 and 4 show Hutchinsonian teeth.

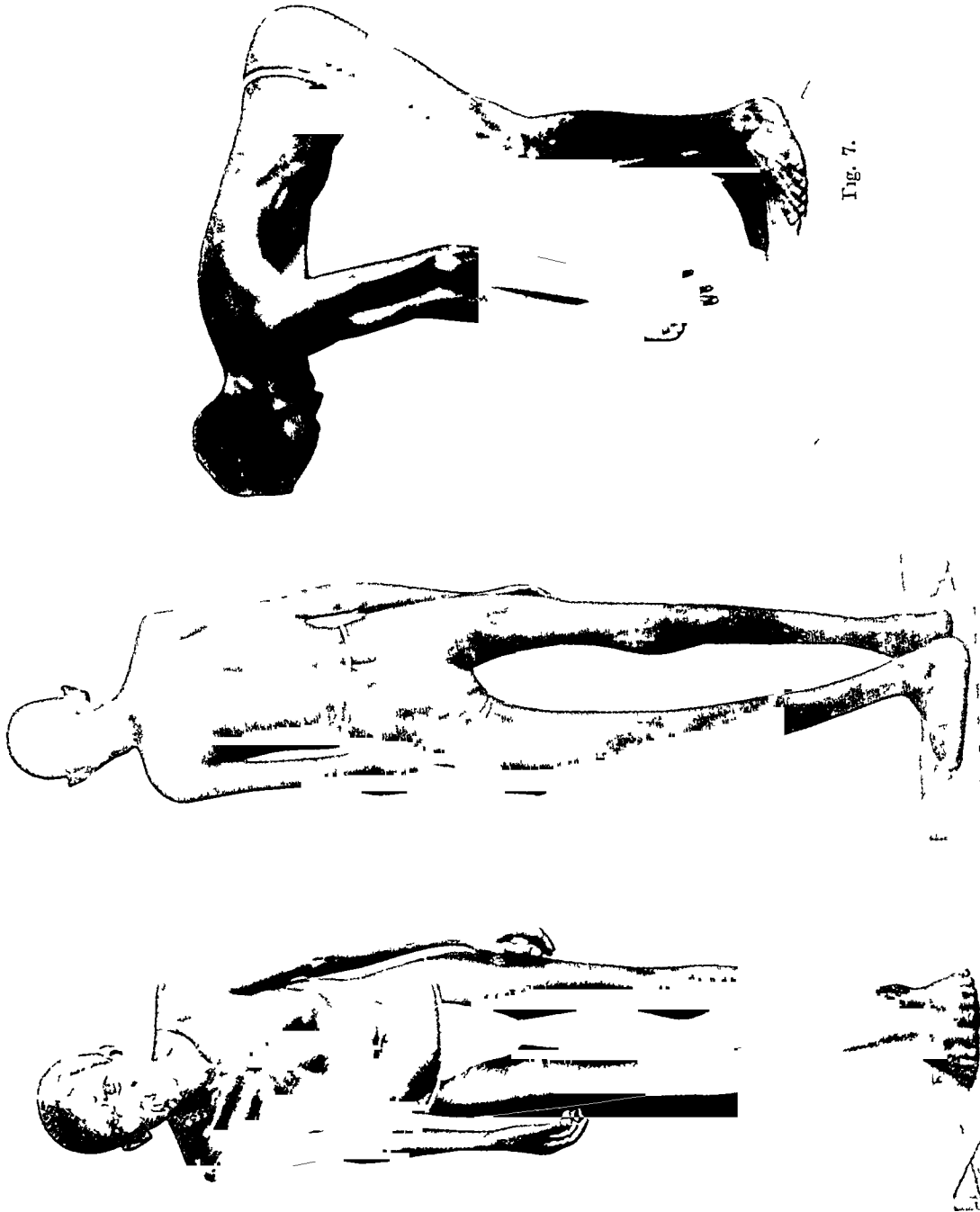
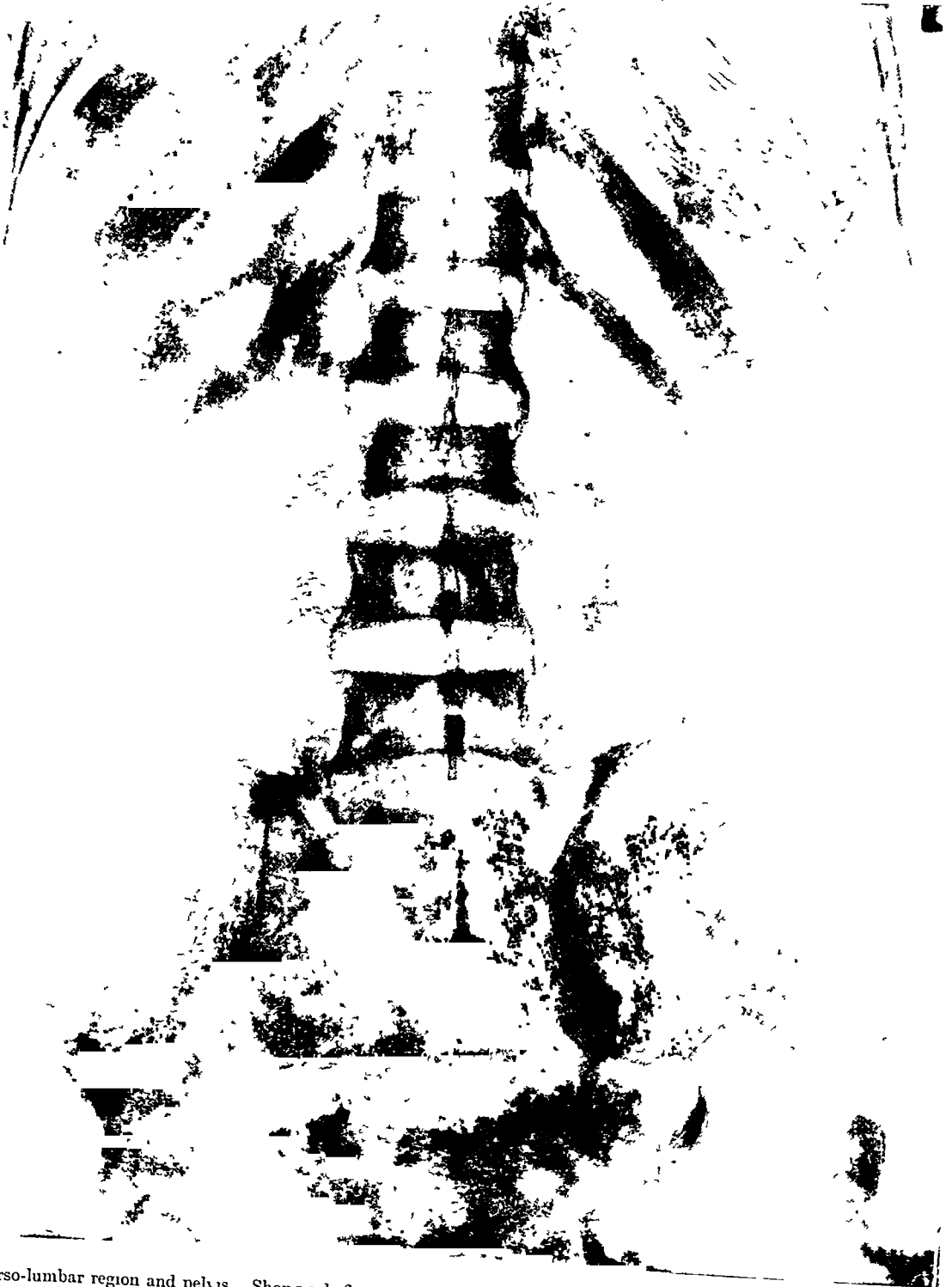


Fig. 5. Stenocostal notches in endemic fluorosis. Fig. 6. Flat chest and stiff intercostal muscles. Fig. 7. Shows rigidity of back. Fig. 8 shows All these show tumoral cachexia.



Dorso-lumbar region of spine showing osteophytic outgrowths of transverse processes and ribs.

PLATE XVI.



Dorso-lumbar region and pelvis. Shows calcification of interspinous ligaments with bridging over of the interspinous spaces, osteophytic outgrowths from transverse processes of vertebrae and from rib margins. The lower outlet of the pelvis is completely bridged by osteophytic outgrowths.

PLATE XIX.



Fig. 10.



Fig. 11.

Fig. 10, foot and ankle. Shows calcification of tendo achilles and irregularities around margin of os calcis. A partly calcified guinea-worm is also shown, internal to the achilles tendon. Fig. 11, radius and ulna with elbow and wrist joints. Shows marked osteophytic outgrowths from radius and ulna along interosseous membrane.

Fluorine in blood and urine.

The method used in estimating fluorine in blood and urine was the Zirconium nitrate-sodium alizarin sulphonate indicator method. As previously stated, our results in these estimations are given with some reserve.

Urine.—In six cases the amount of fluorine in the urine was much above the normal figure.

Blood.—In only three cases was fluorine demonstrated in the blood.

TABLE I.

Blood sugar and blood cholesterol in chronic fluorine poisoning.

Serial number.	Name, etc.	Blood sugar, mg. per cent.	Blood cholesterol, mg. per cent.
I.	Pulipadu Koti Reddi, male, 25 years ..	79.5	159.0
II.	Pulipadu Badull Sahib, male, 35 years ..	95.0	159.0
III.	Baptistpalam Thirupala, male, 30 years ..	107.3	175.5
IV.	Katur Venkitasubbayya, male, 45 years ..	68.8	167.2
V.	Pulipadu Kondigadu, male, 45 years	136.0	174.8
VI.	Desari Pichi, female, 45 years	82.6	165.0
VII.	Pulipadu Guru Vayya, male, 45 years ..	143.3	152.2
VIII.	Desari Venkita Gadu, male, 40 years ..	107.7	168.4
IX.	Katur Venkitasubbamma, female. 35 years ..	68.0	223.2
X.	Parvatipuram Venkitasubbayya, male, 38 years ..	102.6	130.5
AVERAGE ..		99.08	167.48

TABLE II.

Serum calcium, phosphate, phosphatase, magnesium, sodium, and potassium.

Serial number.	Name, etc.	Serum calcium Ca, per cent.	Serum inorganic phosphorus, p.	Ca × P.	Serum phosphatase, unit.	Serum magnesium, mg. per cent.	Serum sodium, mg. per cent.	Serum potassium, mg. per cent.
I.	Pulipadu Koti Reddi, male, 25 years	12.7	4.8	60.95	0.16	2.16
II.	Pulipadu Badull Sahib, male, 35 years .. .	8.4	7.0	58.80	0.15	2.13
III.	Baptistpalam Thirupala, male, 30 years	9.9	4.2	41.58	0.32	2.27
IV.	Katur Venkitasubbayya, male, 45 years	No serum.		..	0.18
V.	Pulipadu Kondigadu, male, 45 years	11.9	3.61	42.95	0.55	1.88
VI.	Desari Pichi, female, 45 years ..	8.3	6.5	53.95	0.17	1.94	353	29.4
VII.	Pulipadu Guru Vayya, male, 45 years	11.9	3.68	43.78	0.40	1.52	342.6	16.9
VIII.	Desari Venkita Gadu, male, 40 years	10.5	4.5	47.25	0.13	1.48
IX.	Katur Venkitasubbamma, female, 35 years	12.7	5.67	72.01	0.31	2.62	386	21.9
X.	Parvatipuram Venkitasubbayya, male, 38 years	13.3	5.9	78.48	0.29	2.63	324.9	47.9
	AVERAGE ..	11.07	5.09	55.53	0.266	2.07	351.6	29.02

TABLE III.

Kidney efficiency tests.

Serial number.	Name, etc.	Urine in 1 hour, in c.c.	Blood urea, mg. per cent.	Urine urea, mg. per cent.	Clearance, in c.c.	Percentage of average normal.	Blood creatinine, mg. per cent.	Urine creatinine, mg. per cent.	Filtration rate, in c.c.	Blood ure acid, mg. per cent.	Urine ure acid, mg. per cent.	Uric acid clearance, in c.c.	Blood chloride, mg. per cent.	Urine chloride, mg. per cent.	Chloride clearance, in c.c.
I.	Pulipadu Koti Reddi, male, 25 years ..	140.0	38.61	1,600	Max. 96.83	129.0	1.8	178.5	228.1	2.8	25.7	Max. 21.5	497.6	1,670	Max. 7.8
II.	Pulipadu Badull Sahib, male, 35 years ..	56.0	48.5	950	St. 18.3	33.0	2.3	63.98	25.8	2.6	19.5	St. 6.0	431.2	813.0	St. 1.75
III.	Baptistipalam Thirupala, male, 30 years ..	21.0	48.5	2,690	St. 32.8	60.1	2.4	178.5	26.0	3.0	27.2	St. 5.3	522.0	1,012.0	St. 1.15
IV.	Katur Venkatasubbayya, male, 45 years ..	39.0	32.7	2,330	St. 57.5	106.6	No blood.	120.0	..	3.7	12.7	St. 2.23	No blood.	1,787	..
V.	Pulipadu Kondigadu, male, 45 years ..	13.0	46.52	2,520	St. 25.2	46.6	2.0	219.8	24.5	3.7	14.2	St. 5.6	511.6	1,479	St. 1.3
VI.	Desari Pichi, female, 45 years ..	118.0	69.2	80	St. 1.6	2.9	2.1	23.36	22.2	3.7	3.15	St. 1.3	526.0	127.0	St. 1.14
VII.	Pulipadu Guru Vayya, male, 45 years ..	55.0	31.7	810	St. 24.4	45.2	1.4	136.0	87.0	2.19	35.56	St. 13.7	609.8	1,621	St. 2.55
VIII.	Desari Venkita Gadu, male, 40 years ..	171.0	30.69	920	Max. 85.51	114	2.1	88.28	118.5	3.4	13.11	Max. 10.98	558.5	1,256	Max. 6.47
IX.	Katur Venkitasubhamma, female, 35 years ..	74.0	62.34	80	St. 1.38	2.6	1.8	11.11	7.73	2.3	11.77	St. 5.77	590.3	302.2	St. 0.57
X.	Parvatipuram Venkita-subbayya, male, 38 years ..	70.0	81.17	690	St. 9.15	16.94	1.7	51.2	37.0	2.6	12.82	St. 5.3	502.4	832.6	1.79

TABLE IV.

Fluorine in blood and urine and qualitative examination of urine.

Serial number.	Name, etc.	Fluorine in blood, mg. per cent.	Fluorine in urine, mg. per cent.	Urine.	Qualitative.
I.	Pulipadu Koti Reddi, male, 25 years	Albumin. Sugar. Micros.	Nil. " Nothing abnormal.
II.	Pulipadu Badull Sahib, male, 35 years ..	1.80	1.58	Albumin. Sugar. Micros.	Nil. " Bladder Epith. and calcium oxalate.
III.	Baptistpalam Thirupala, male, 30 years	Albumin. Sugar. Micros.	Nil. " Triple phosphate.
IV.	Katur Venkitasubbayya, male, 45 years	1.80	Albumin. Sugar. Micros.	Nil. " Nothing abnormal.
V.	Pulipadu Kondigadu, male, 45 years ..	1.80	..	Albumin. Sugar. Micros.	+ + Bladder Epith. and calcium oxalate.
VI.	Desari Pichi, female, 45 years ..	1.85	1.65	Albumin. Sugar. Micros.	+ Nil. Nothing abnormal.
VII.	Pulipadu Guru Vayya, male, 45 years	4.08	Albumin. Sugar. Micros.	Nil. Trace. Much Epith. debris and leucocytes.

TABLE IV—*concl'd.*

Serial number.	Name, etc.	Fluorine in blood, mg. per cent.	Fluorine in urine, mg. per cent.	Urine.	Qualitative.
VIII.	Desari Venkita Gadu, male, 40 years	Albumin. Sugar. Micros.	Nil. " Triple phosphate and bladder Epith.
IX.	Katur Venkitasubamma, female, 35 years	1.35	Albumin. Sugar. Micros.	Nil. " Amorphous debris.
X.	Parvatipuram Venkita-subbayya, male, 38 years	..	1.50	Albumin. Sugar. Micros.	Nil. " Triple phosphate.

SUMMARY.

1. Ten cases of chronic fluorine intoxication have been investigated, clinically, radiologically, and, as regards blood and urine, biochemically.

2. The clinical picture is described and relates chiefly to disabilities caused by calcification of ligaments, tendons and fasciæ, the formation of osteophytic outgrowths of bone and the nervous effects of mechanical pressure by encroachment of bone on the spinal canal.

3. The radiological findings show excessive calcification of tendons, ligaments and fasciæ, the production of osteophytic formations from various bones, and the almost complete synostosis of various joints, especially those of the vertebral column. These give the clue to the clinical findings.

4. The biochemical estimation of serum calcium, inorganic phosphate, and serum phosphatase indicates a favourable condition for abnormal deposition of bone.

5. Kidney function in the majority of the cases is impaired.

6. The urine contains amounts of fluorine much above the normal upper limit.

ACKNOWLEDGMENTS.

We wish to express our indebtedness to Dr. J. Lakshminarayana, the District Health Officer, Nellore, for his assistance in procuring the cases for admission into hospital in Madras.

In the clinical examination of the cases we were fortunate in having the services of Dr. D. Govinda Reddy, Assistant to the Professor of Medicine, who spared no time and trouble in his investigations.

To the Medical and Lay staff of the Barnard Institute of Radiology, Madras, we owe a debt of gratitude for the skill and painstaking care expended by them on an extremely thorough radiological investigation of our material.

REFERENCES.

Note.—As the condition now described has not previously been noted in India, we had prepared an extensive series of references. Since the completion of our manuscript, however, we have received the very comprehensive memoir on fluorosis by K. Roholm entitled 'Fluorine Intoxication'. This contains so complete a bibliography on this subject that it would be redundant for us to give our list and we would refer those interested to the work mentioned. In order, however, that workers on this subject may have as complete a list of references as possible, we have given 63 references out of the list prepared by us, which were not contained in Roholm's bibliography.

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STUDIES ON THE SPECIFIC POLYSACCHARIDES OF THE VIBRIOS.

Part I.

THE EFFECT OF THE GROWTH-MEDIUM.

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DURING our study of the vibrios it has become increasingly clear that the medium in which they are grown has a profound effect both on the quality and on the quantity of the vibrio polysaccharide. This finding has now been investigated in some detail by culturing a given strain on a number of media, and preparing the polysaccharides by a standardized technique. The present paper reports the comparative serological and chemical study of these preparations.

METHODS.

The vibrio strain chosen for these experiments had been found to give good yields of polysaccharide in previous work. It belonged to chemical group VI, and contained protein I and polysaccharide III (Linton, Shrivastava and Mitra, 1935). It was grown for about 72 hours at 37°C. in 21 litres of each of the following media, which had a pH of 8.0 before the final filtration and autoclaving:—

1. Merck's peptone, 10 g.; NaCl, 5 g.; distilled water, 1 litre.
2. Bactopeptone, 10 g.; NaCl, 5 g.; distilled water, 1 litre.
3. Papain digest mutton broth, 1 litre (containing 1 per cent oxidizable matter); NaCl, 5 g.
4. Mutton infusion broth, 1 litre; Merck's peptone, 10 g.; NaCl, 5 g.

5. Merck's peptone, 10 g.; Na_2HPO_4 , $12\text{H}_2\text{O}$, 21.6 g.; NaH_2PO_4 , $2\text{H}_2\text{O}$, 1.04 g.; NaCl, 5 g.; distilled water, 1 litre.
6. Identical with 5, and containing in addition 3 g. glucose per litre.
7. Merck's peptone, 10 g.; NaCl, 5 g.; NaH_2PO_4 , $2\text{H}_2\text{O}$, 8 g.; NaOH, 1.7 g.; distilled water, 1 litre.
8. Identical with 7, and containing in addition 3 g. glucose per litre.

PREPARATION OF THE POLYSACCHARIDES.

After 72 hours' growth, phenol was added to the media to make a concentration of 0.5 per cent and the culture flasks were well shaken and allowed to stand for 30 minutes. The fluid was then freed of organisms by passing it through a Sharples' supercentrifuge, and the clear centrifugate reduced to one-tenth its volume by concentration *in vacuo* at a water-bath temperature of about 55°C . The method then followed was similar to that already published (Shrivastava and Seal, 1937), although it may be pointed out that minor modifications were sometimes necessary depending upon which medium had been used for growth and the concentration of the polysaccharide. When media containing phosphates were used the amount of alcohol required to precipitate the polysaccharide from the concentrated fluid was between 1.25 and 1.5 volumes in the presence of 3 per cent sodium acetate and 1.5 per cent glacial acetic acid, whereas without the phosphates two volumes or more of alcohol were required. On centrifuging after precipitation, three layers were obtained. The top layer consisted of alcoholic supernatant, the middle layer was a solid precipitate containing almost all the polysaccharide, and the bottom layer was a syrupy deposit of phosphates. The middle layer alone was used, the others being discarded. It was taken up in 400 c.c. of water together with 16 g. of sodium acetate and 8 c.c. of glacial acetic acid and after being stirred in a mechanical stirrer it was centrifuged. Usually one such extraction was enough to dissolve out all the active substance from the crude precipitate in the middle layer. The solution was precipitated with one volume of alcohol two or three times, and the final precipitate was dissolved in 400 c.c. of water containing 16 g. of sodium acetate and 8 c.c. of glacial acetic acid. Forty c.c. of chloroform and 4 c.c. of either n-butyl alcohol or amyl alcohol were added and the whole shaken in a mechanical shaker for one and a half hours and then centrifuged. The precipitated protein was removed and the process repeated until the amount of protein falling out each time was negligible. Usually four to six such shakings were necessary. Further precipitations with glacial acetic acid and alcohol followed and the final isolation of the polysaccharide was made by precipitating it with redistilled alcohol in the presence of sodium acetate, and washing it with redistilled alcohol followed by two washings with acetone. After filtering it under suction it was dried *in vacuo* at 61°C ., the boiling point of chloroform.

CHARACTERISTICS OF THE POLYSACCHARIDES.

All the polysaccharides were free from glycogen, starch, and phosphates as well as from protein, as shown by the biuret reaction. They gave a Molisch reaction at a dilution of one million times. The polysaccharides could be roughly divided into two classes, those showing homologous precipitin titres of several

millions and those showing titres of only several thousands. The former on drying resembled matted fibres of dried filter-paper. They dissolved in water only with difficulty and gave solutions of high viscosity. The other group were amorphous polysaccharides on drying and gave a perfectly clear solution of no obvious viscosity. It is probable that this differentiation is bound up with the length of the chains of the molecules which make up the polysaccharide solution: when these are long, fibrous polysaccharides and viscous solutions are produced which have high serological reactivity; where the chains are short, the dried polysaccharides are amorphous, do not form viscous solutions, and react less strongly with the antiserum.

The chemical properties of these polysaccharides are compared in Table I, in which the data are arranged in the ascending order of their reactivity with the homologous antiserum in the precipitin test:—

TABLE I.

Chemical and serological properties of the polysaccharide of a vibrio strain grown on various media.

Number.	Medium number.	Yield per litre, mg.	Ash, per cent.*	Specific rotation, $[\alpha]_D^{\dagger}$	N, per cent.†	Acetyl, per cent.†	Maximum hydrolysis, per cent.†	Homologous precipitin titre.
1	8	1.8	..	+ 60.1	0.81	..	53.0	2.4×10^3
2	3	5.1	..	+ 32.6	1.85	5.5	41.0	9.6×10^3
3	6	1.8	..	+ 75.0	0.83	..	70.0	1.9×10^4
4	3	4.8	..	+ 6.3	2.65	..	46.0	1.5×10^5
5	2	3.4	0.21	..	43.2	6.1×10^5
6	1	3.0	..	+ 58.1	1.15	..	57.0	2.5×10^6
7	5	3.2	5.9	+ 62.6	1.81	8.5	56.7	4.0×10^6
8	7	9.1	6.0	+ 46.8	3.28	13.4	69.9	8.0×10^6
9	1	8.2	6.8	+ 56.0	3.40	9.9	62.6	10.0×10^6
10	1	14.1	7.8	+ 58.0	2.62	8.3	62.2	12.0×10^6
11	4	16.4	5.9	+ 53.0	3.16	12.8	65.8	16.0×10^6

* As Na_2O .

† Calculated on an ash-free basis.

Hydrolysis was carried out in N/1 H_2SO_4 in sealed tubes in the boiling water-bath. The estimations of reducing substance were made by the Hagedorn and Jensen method, and are expressed in terms of glucose.

The strain used in these experiments has in general opaque colonies when grown on agar plates. Occasionally, however, a transparent colony appears. Preparation No. 2 was obtained from the growth of such a colony and is somewhat less active serologically and has a different specific rotation than the opaque strain grown in the same medium (preparation No. 4). In the same way, preparation No. 6 represents the growth of a transparent colony, and has given a much smaller yield than preparations Nos. 9 and 10 which are from opaque colonies on the same medium.

Polysaccharide yield is best in plain mutton infusion broth with peptone (No. 4), both in quantity and in quality, as shown by the precipitin titre. Next in order are Merck's peptone alone (No. 1), and Merck's peptone with NaOH-phosphate buffer (No. 7). The double phosphate buffer with Merck's peptone (No. 5) gives a poor yield, although the reactivity of the product is high. With media Nos. 6 and 8, which are identical with Nos. 5 and 7, respectively, except that they contain 0.3 per cent glucose, both the quality and the quantity of polysaccharide are much reduced. With bactopeptone (No. 2) only a small amount of moderately active polysaccharide is produced, and with papain digest broth (No. 3) somewhat larger amounts of poor precipitating power are found.

Since the antiserum is capable of reacting with the polysaccharide dilutions up to 16,000,000, it is clear that reactions of only several thousands are to be regarded as practically negative, and in these experiments low yield was in general accompanied by low reactivity. That the two are not entirely correlated is shown, however, by some unpublished work in which relatively large amounts of inactive polysaccharide were obtainable from other vibrio strains.

It is interesting to compare the yields of polysaccharide with the age of the strain. For example, experiment No. 10 in which a yield of 14.1 mg. per litre was obtained was done a year earlier than experiment No. 9 in which the yield was only 8.2 mg. per litre, and accordingly between the two experiments the strain had undergone a year of artificial cultivation, and it is possible that such long-continued artificial cultivation lessens the strain's capacity to produce polysaccharide. We have noted that some other old strains show only very small amounts of specific substance.

The figures for ash, which average about 6 per cent expressed as Na_2O , are rather high, but were not unexpected as the polysaccharides were isolated as the sodium salts. In the highly reactive polysaccharides the specific rotations lie between $+53.0^\circ$ and $+62.0^\circ$. With the less active polysaccharides the rotations are irregular. In the active polysaccharides nitrogen appears to be about 3 per cent. In earlier work this figure was found to average about the same, and it was shown that in all types of vibrio polysaccharide some of the nitrogen was present in the amino form, that is, it formed an integral part of the polysaccharide complex (Linton and Mitra, 1936). In general, the preparations having low nitrogen values have low precipitin titres as well, and this may indicate again that a part at least of the total nitrogen is essential for the existence of an intact and highly reactive polysaccharide.

The significance of the figures for acetylation is not yet clear. The figures for percentage acetylation are higher than those previously obtained (Linton and Mitra, *loc. cit.*), as the polysaccharide now being prepared is in a state more nearly approaching that in which it exists in the vibrio. A study is being made of a possible correlation between the amount of acetylation and the serological reactivity of the polysaccharide.

In the first four preparations in Table I the presence of acids in the growth media may have been the cause of the inferior yields and reactivities of the polysaccharides. Some unpublished experiments have shown that typical vibrios are strong producers of both volatile and non-volatile organic acids in sugar media. In unbuffered solutions of glucose, they are able to increase the acidity

a hundredfold in three or four hours. The volatile acids consist of about equal quantities of formic and acetic acids, and the non-volatile acids have not been wholly identified, although there is a strong probability that lactic is one of them. Variations in acidity during growth of the vibrio in the various media used in this work have been followed, and the results have been given in Table II:—

TABLE II.

Changes in pH brought about by a vibrio strain in various media.

		Start.	6 hours.	24 hours.	48 hours.	72 hours.
1. Merck's peptone ..		7.6	7.28	7.68	7.95	8.14
2. Bactopeptone ..		7.8	7.38	7.69	7.97	8.24
3. Papain broth ..		7.62	7.07	7.08	7.02	7.03
4. Broth and peptone ..		7.56	7.07	7.66	8.22	8.43
5. Peptone and double phosphate buffer.		7.8	7.79	7.86	7.9	8.03
6. Ditto with glucose ..		7.6	6.81	6.20	6.24	6.28
7. Peptone and NaOH-phosphate buffer.		7.85	7.78	7.9	8.07	8.19
8. Ditto with glucose ..		7.5	6.62	6.01	6.02	6.09

In all the media there is an increase in acidity during the first six hours of growth. Except in three instances, however, acidity has again been lessened at the 24-hour period, and at 48 and 72 hours the acidity is definitely lower than it was in the original media previous to inoculation, due to the formation of ammonia from the peptone. In media Nos. 3, 6, and 8, however, the acidity does not become less after six hours but goes on increasing until it reaches a constant level. The first of these media contains considerable muscle sugar, and the other two contain glucose, and it is precisely in these media that the polysaccharide is lowest both in quantity and quality. The final acid increase is about 5 times in the papain broth and from 12 to 15 times in the media containing glucose. It is highly probable that this considerable increase in acidity has a deleterious effect on the polysaccharide, which we know to be a labile complex.

It should also be pointed out in this connection that the amounts of growth obtained are not apparently different in any of the media. All gave heavy growth in 24 hours, and did not increase very much thereafter. Nor did the agglutination test vary according to the medium and the amount of precipitin activity of the polysaccharide, but in each case the strain agglutinated to titre with the homologous antiserum, irrespective of the quantity or quality of the polysaccharide which could be isolated.

DISCUSSION.

From the data we have presented it is evident that the type of medium used has a profound effect on the production and character of the specific polysaccharide. We believe that it is possible to rule out the effect of technique as the cause of this variation. The method has been uniform throughout in all essential details, and in no case were drastic methods of heating or treatment with alkali or mineral acids used, which might account for loss of activity and yield in some of the preparations.

Glucose fermentation appears to be the principal factor at work in lowering yield and serological activity. The active production of organic acids from glucose which is a marked feature of vibrio metabolism probably brings about changes in the polysaccharide, which either break it down or make its isolation impossible with present methods. In either case it would appear that the polysaccharide undergoes changes which lower its serological activity. It may also be pointed out that the results given in this paper show that unless conditions of growth are standardized, comparisons of polysaccharide yields and the reactivities of different strains are without much significance.

SUMMARY.

A vibrio has been grown on eight different liquid media and polysaccharides prepared from the clear centrifugate by a method in which the use of alkalis, mineral acids, and other drastic means of preparation are carefully avoided.

The polysaccharides from different media differ greatly in physical and chemical properties as well as in their reactivity in the precipitin test, although they were all derived from the same organism and tested against the same homologous serum.

The poorest yields and the least active polysaccharides were obtained in media containing glucose, and it is suggested that the active production of organic acids in such media may account for the results.

Polysaccharide prepared from growth in mutton infusion broth made up with 1 per cent Merck's peptone gave the best yield and the highest reactivity, viz., 16.4 mg. per litre and a reaction at a dilution of 1 : 16,000,000.

The pH determinations in Table II were done with a quinhydrone electrode by Mr. S. N. Mukerji, M.Sc., of the Department of Pharmacology of the School of Tropical Medicine, Calcutta, to whom our thanks are due.

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 SHRIVASTAVA, D. L., and SEAL, S. C. (1937) *Proc. Soc. Exper. Biol. & Med.*, **36**, p. 157.

CHEMICAL AND SEROLOGICAL VARIATION IN SINGLE-CELL CULTURES OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS.

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IN our previous communications (Linton, Shrivastava and Mitra, 1935 ; Linton, Mitra and Seal, 1936) we have indicated the four ways in which we have observed the vibrios and vibrio strains to vary, namely, by loss or gain in the proportions of a constituent (as in the smooth-rough transition), by the complete replacement in a cell of a constituent part by another of a different chemical constitution, by the presence in a strain of organisms possessing more than one type of chemical structure, in which case the variation consists in changes in the proportions of the two constituent organisms, and finally by changes in the surface electric charges. Although we have found variation from one to another of the six chemical groups to be of fairly frequent occurrence under laboratory conditions, we could not be sure, when working with strains isolated in the ordinary way, that we were not dealing in every case with cultures originally mixed and in which the common phenomenon of replacement of one type by the other was not occurring. In order to avoid this difficulty we have now studied variation in cultures descended from single cells, and it has been found that the same shifts in the chemical groups occur in these as in the cultures previously studied. The purpose of this paper is to present these results.

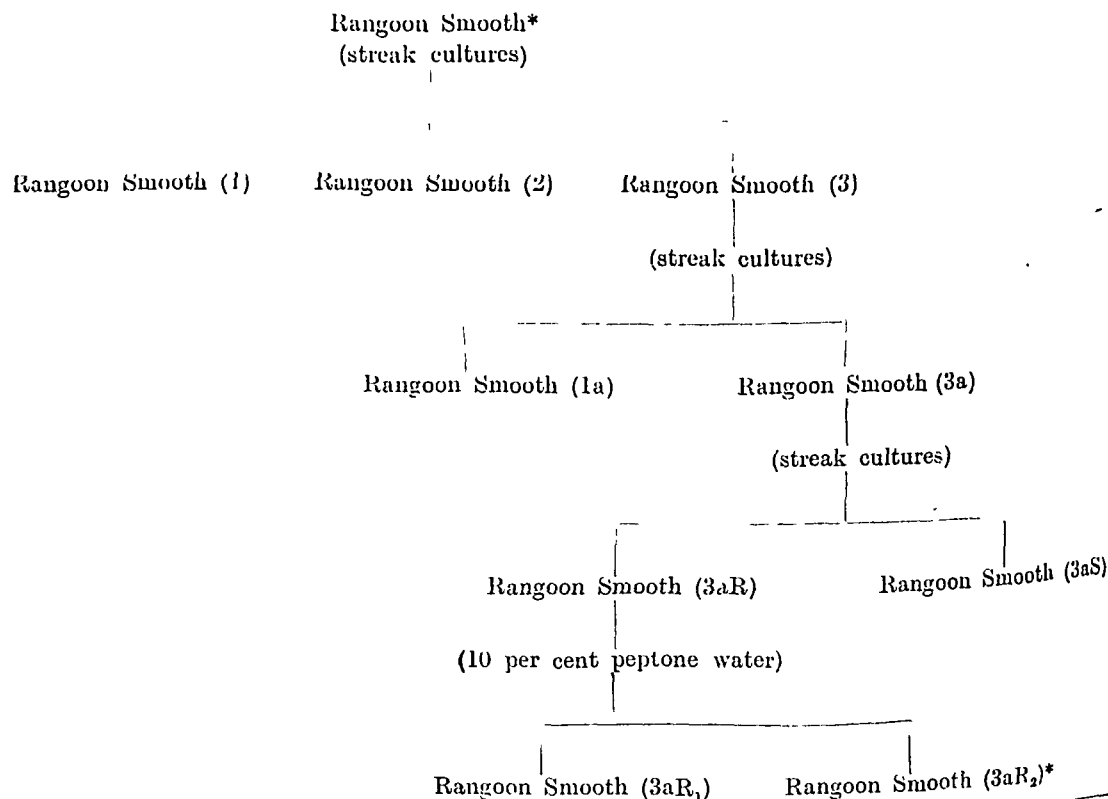
METHOD.

Single-cell cultures of the two strains, Rangoon Smooth and Rangoon Rough (2), were kindly isolated for us by Dr. C. G. Pandit of the King Institute, Madras, by the micro-pipette method, and strain X (1) was isolated in Shillong by the same method. We have isolated two single-cell strains, 1617 and Inaba, by the darkground illumination method described by Topley, Barnard and Wilson (1921) and used by one of us in work on the vibrios (Seal, 1937). The same results have been obtained with strains isolated in either way.

The general methods used for isolating variants from these cultures are outlined in Table I. The essential part of the method was to make repeated streak cultures, picking up in each case the colony or portion of the colony showing the characteristics desired, these being roughness in the case of the Rangoon Smooth culture and smoothness in the case of the Rangoon Rough (2) culture. In addition, the partially rough variant of the smooth culture was passed repeatedly through 10 per cent peptone water and the partially smooth variant was passed likewise through 0.5 per cent glucose broth for ten days, as previous experience had shown that these methods are very useful in bringing about the desired transitions.

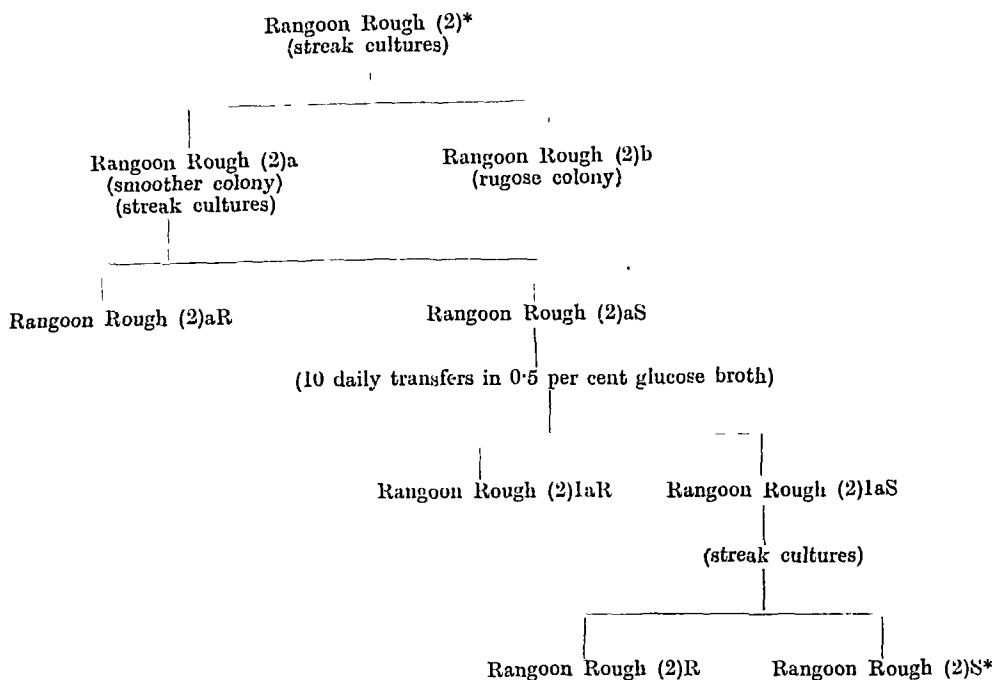
TABLE I.

Mode of derivation of cultures from single-cell strains of Rangoon Smooth.



* The characteristics of these two strains are compared in Table II.

TABLE I—concl'd.

Mode of derivation of cultures from single-cell strains of Rangoon Rough (2).

	'RANGOON SMOOTH SINGLE CELL.'		'RANGOON ROUGH (2) SINGLE CELL.'	
	'R. S. S. C.' original.	'R. S. S. C.' (3aR ₂).	'R. R. (2) S. C.' original.	'R. R. (2) S. C. S.'
Colonies on agar	Smooth, translucent.	Rough, opaque.	Dry, rugose.	Smooth, translucent.
Morphology	Typical and short forms.	Slightly longer and thin curved form.	Thick small rods, almost bacillary shape.	Typical but thin.
Flagella	Single terminal.	Single terminal.	Single terminal.	Single terminal.
Motility	Actively motile.	Actively motile.	Actively motile.	Highly motile.
Peptone water, 24 hours' growth	Uniform turbidity, faint pellicle and no deposit.	Thin turbidity, thick pellicle and deposit.	Thin turbidity, thick pellicle and deposit.	Uniform turbidity, thin pellicle and no deposit.
Indol reaction	Positive.	Positive.	Negative.	Positive.
Reaction with Millon's reagent	Sr.	R.	R.	Sr.
Sugar fermentation reaction (Heiberg).	I	I	V	I
Proteolysis (gelatin) ..	Positive (sl.).	Positive.	Negative.	Positive (sl.).
Hæmolytic power (sheep cells)	Negative.	Trace.	Trace.	Negative.
Salt stability (physiological salt solution).	Stable.	Unstable (partially).	Unstable (partially).	Stable.

II.

1617 'SINGLE CELL'.		INABA 'SINGLE CELL'.		'SHILLONG X (1) SINGLE CELL.'	
1617 'S. C.' original.	1617 'S. C.' R.	Inaba 'S. C.' original.	Inaba 'S. C.' R.	'Shillong X (1) S. C.' original.	'Shillong X (1) S. C.' R.
Smooth, translucent.	Rough opaque.	Smooth translucent.	Rough, opaque.	Smooth, translucent.	Rough, opaque.
Typical but slightly short.	Long and thin form.	Typical but short.	Short and thick and long and thin forms.	Typical and short forms.	Thin and long forms and chains.
Single terminal.	Single terminal.	Single terminal.	Single terminal.	Single terminal.	Single terminal.
Actively motile.	Sluggishly motile.	Motile.	Motile.	Sluggishly motile.	Sluggishly motile.
Uniform turbidity, thin pellicle and no deposit.	Very thin growth, thick pellicle and slight deposit.	Uniform growth, thin pellicle and no deposit.	Thin growth, thick pellicle and deposit (sl.).	Uniform turbidity, thin pellicle and no deposit.	Thin growth, thick pellicle and slight deposit.
Positive.	Positive.	Positive.	Positive.	Positive.	Positive.
S.	R.	S.	R.	Sr.	R.
I	II	I	I	I	I
Positive.	Positive.	Positive.	Positive.	Positive.	Positive.
Negative.	Trace.	Negative.	Positive (sl.).	Negative.	Positive (sl.).
Stable.	Unstable.	Stable.	Unstable.	Stable.	Unstable (partially).

CULTURAL AND BIOCHEMICAL CHANGES IN VARIANTS DERIVED FROM
SINGLE-CELL CULTURES.

The most marked change occurred during the variation of strain Rangoon Rough (2) to give rise to a smooth strain. The original single-cell culture had the characteristics of its parent strain: dry, rugose colonies; thick pellicle formation, a deposit, and a clear central portion of the broth; a negative indol reaction; fermentation of the significant sugars according to Heiberg's group V; and unstable both when heated with Millon's reagent and in normal salt solution. The derived strain, on the other hand, grew in smooth, translucent colonies; in broth showed the typical uniform turbidity and thin pellicle formation of the smooth vibrios; produced indol; fell into Heiberg's group I in respect to sugar fermentation; and was perfectly stable both to Millon's reagent and in normal saline.

The other variants showed changes of the same kind, although they were not so complete as in the case of Rangoon Rough (2).

CHANGES IN CHEMICAL CONSTITUTION.

The changes which took place both in polysaccharide and protein constitution during variation are outlined in Table III:—

TABLE III.

Chemical composition of original and variant single-cell vibrio strains.

			Protein.	Polysaccharide.	Chemical group.
Rangoon Smooth	..	Original	I	III	VI
		Variant	II	I and III	IV and V
Rangoon Rough (2)	..	Original	II	III	V
		Variant	I	I	I
1617	..	Original	I	III	VI
		Variant	II	I and III	IV and V
Inaba	..	Original	I	I	J
		Variant	II	I and II	III and IV
Shillong X (1)	..	Original	I	III	VI
		Variant	II	I and III	IV and V

For the differentiation of protein I and protein II, see Linton, Mitra and Shrivastava (1934).

From these data, it is evident that in each case during variation the proteins have changed from one to the other type, as shown by their racemization curves (Linton, Mitra and Shrivastava, 1934). In the case of strain Rangoon Rough (2) and its variant there has also been a complete shift in polysaccharide, the former containing the glucose type and the latter the galactose type. In the other cases the variants' polysaccharides are mixtures of the originally present polysaccharide and a new one. In these latter instances it is possible that variation was still in progress and the derivative chosen belonged to a stage at which it was not yet completely shifted from one polysaccharide to the other. Further work on these cultures would probably have permitted the isolation of strains which had completely varied in respect to their polysaccharide structure. This assumption is supported by the evidence from serology given below.

It is clear that starting with a single-cell culture having a given protein and polysaccharide constitution, it is possible to induce variations which yield a strain having a constitution of a different type, and the variant strain then falls into another chemical group.

VARIATIONS IN SEROLOGICAL REACTIONS.

Agglutination reactions with homologous and heterologous antisera have been done at various stages of variation, and the results of some of these tests are shown in Table IV :—

TABLE IV.

Variations in homologous agglutination titres during dissociation in single-cell cultures of the Rangoon strains.

			Rangoon Smooth serum ('H' and 'O'). Titre 1 : 6,400.	Rangoon Rough (2) serum ('H' and 'O'). Titre 1 : 12,800.
Rangoon Smooth—				
Original	..		6,400	0
1	400*	200
3	400*	400*
(3aR ₂)	..		400	6,400
Rangoon Rough (2)—				
Original	..		0	12,800
a	0	12,800
1aS	.		1,600	200
S	..	.	6,400	0

* Maximum dilution used.

Variations in standard Inaba 'O' serum during dissociation in single-cell cultures of the Rangoon strains.

		Inaba 'O' serum. Titre 1:2,500.
Rangoon Smooth—		
Original	.	2,500
3aR ₂	..	200
Rangoon Rough (2)—		
Original	..	0
S	2,500

The numbers of the cultures in this table indicate the stage of derivation according to the scheme in Table I. The results indicate that during variation there is a gradual loss of agglutinability of the two strains with their own antisera, and this change is paralleled by a gradual increase in agglutinability with the antisera of the other strain, until at the end of the process the position of these two strains and their antisera has become reversed. The process with Rangoon Rough (2) went completely to an end and its smooth derivative did not agglutinate at all with the original antiserum. In the case of Rangoon Smooth the change was not quite complete, and agglutination with the homologous antiserum still occurs, but to only about 5 per cent of the titre.

The same findings were made when the originals and derivatives were tested against the standard agglutinating Inaba 'O' serum. Rangoon Smooth agglutinates to titre while Rangoon Rough (2) is negative. In their derivatives, on the other hand, the position is again reversed. Rangoon Smooth (3aR₂) agglutinates to less than 10 per cent of titre, and Rangoon Rough (2) S agglutinates to full titre.

The same general tendency is shown in the original and derived strains of Inaba and 1617 (Table V). In this case the cross reactions of the two strains were also done, and show that with either serum practically the same result is obtained, and the variant strains agglutinate to only a small fraction—6 per cent to 12 per cent of titre.

TABLE V.

Variations in agglutination titres during dissociation in single-cell cultures of vibrio strains 'Inaba' and 1617.

	Inaba serum 1 : 6,400.	1617 serum 1 : 6,400.
Inaba—		
Original .	6,400	6,400
Variant .	400	800
1617—		
Original ..	6,400	6,400
Variant ..	800	400

DISCUSSION AND SUMMARY.

The work reported in this paper represents a further study of the basis of variation in the vibrios. The evidence in the case of the single-cell cultures indicates a chemical basis for the variations we have observed. We began in each case with a culture descended from a single cell and having a certain set of characteristics: biochemical, cultural, serological; and a certain chemical structure. At the end of the experiment, we had produced from this culture a new strain having another set of biochemical, cultural and serological characteristics, and a different chemical structure. It followed that the new strain fell into a different chemical group than the old, and we wish to emphasize strongly that all the characteristics of the new strain were similar to those of other strains in the chemical group into which it now fell. In other words, the changes in chemical composition, biochemical reactions, cultural and serological properties are correlated.

It is of interest to note further that while the vibrios vary in this correlated way, they always remain within the framework of the six chemical groups found in our previous studies, that is, the framework of the two proteins and the three polysaccharides. Within these limits the powers of synthesis and variation are considerable but the organisms appear incapable of giving rise to any other chemical constituents. The conception is that of a strictly limited capacity for transformation.

Although almost nothing is known about the internal arrangements of the vibrios, it may perhaps be permissible to suggest that each of them possesses the enzymic equipment capable of synthesizing the various proteins and polysaccharides which are found in the whole group ; that during the course of synthesis and growth sometimes one and sometimes another of these constituents is formed ; and that as a result of this shifting in the direction of synthesis a new type of organism is produced which gives rise to a strain different in character because it is different in chemical composition from the original strain. Since in the present experiments all the cells were descended from one cell, and yet were capable of giving rise to variants, it is difficult to account for our observations on any other basis.

ACKNOWLEDGMENTS.

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AN EPIDEMIC OF CHOLERA IN A RURAL AREA IN SOUTH INDIA CAUSED BY THE 'OGAWA' TYPE OF *V. CHOLERÆ*.

BY

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TAKAGI and MURAYAMA were the first to demonstrate serological types in *V. cholerae* by means of agglutination and absorption tests (Nobechi, 1933). Kabeshima (1913—quoted by Takano *et al.*, 1926; 1918) showed that the cholera vibrios isolated during the epidemic of 1912 in Japan were immunologically different from the vibrios isolated from sporadic cases occurring in Formosa in the same year, and designated these two types as 'original' and 'varied' respectively. Nobechi (1923) confirmed the presence of these two types and added a third which he called 'type intermediate'. These observations were made before the differentiation of the heat-labile and heat-stable antigens of the vibrios of the cholera group by Balteanu (1926), Shousha (1931), Abdoosh (1932), Gohar (1932), and the demonstration of the non-specific nature of the thermolabile antigens by Shousha (*loc. cit.*), Abdoosh (*loc. cit.*), Bruce-White (1934), the latter phenomenon being confirmed by Gardner and Venkatraman (1935*a*), who demonstrated a great diversity of heat-stable 'O' antigens among 101 vibrio strains which they examined. Gardner and Venkatraman also suggested that, as a working rule, only non-hæmolytic vibrios of their 'O' sub-group I should be accepted as true *V. cholerae*. They further confirmed the presence of two serological types within the classical sub-group (Gardner and Venkatraman, 1935*a, b*) and designated them as types 'Inaba' and 'Ogawa' after the type cultures received from Japan.

Observations on the types of vibrios in relation to definite epidemics are scanty. A review of the Japanese work leaves one with the impression that the

'original' or 'Inaba' type is associated with epidemic outbreaks and severity of infection and 'varied' or 'Ogawa' type with sporadic cases and mild outbreaks. Manako (1933), however, records that 187 strains of cholera vibrios examined during the outbreak of cholera in Manchuria in 1932 were for the most part of the 'varied' (Ogawa) type.

Early in the course of a serological examination of strains of *V. cholerae* isolated from different parts of the Madras Presidency in 1936, it was noticed that all strains derived from Periyakulam taluk of the Madura district were of the 'Ogawa' type. The course of the epidemic in the Periyakulam health range was closely followed throughout the year 1936 and vibrios were isolated at different periods and from the several villages of the range with a view to studying the relation of the type of vibrio to the nature of the epidemic.

Periyakulam range is a small rural tract in the west of Madura district with an area of about 400 square miles and a population of nearly 200,000. A part of it is hilly, but the greater portion lies in the plains and is freely watered by the tributaries of the Vaigai. This area was entirely free from cholera during 1933, 1934 and 1935. While there was very little cholera anywhere in the district as a whole during 1933 and 1934 (32 attacks in 1933 and 9 in 1934), there were 2,116 attacks in parts of the district other than the Periyakulam range in 1935.

The first village in the range to report cholera was Melmangalam where, between 20th January, 1936 and 6th February, 1936, there were 16 attacks and 7 deaths. Specimens from two cases were submitted for bacteriological examination and *V. cholerae*, type 'Ogawa', was isolated from both. From Melmangalam the infection spread to the other villages of the range and the epidemic continued throughout the year with a break of three weeks in April, till the end of the year by which time nearly every village and hamlet had been involved.

In 39 villages with a population of 181,668 there were 2,839 attacks and 1,257 deaths from cholera during the year, giving an attack rate of 15.6 per mille and a death rate of 6.8 per mille. These figures have been the highest for several years, the mean annual mortality from cholera for 60 years ending 1927 in the Madura district being below 2.5 per mille. Only in 6 years in this period has the mortality exceeded 5 per mille (Russell, 1929). The case mortality of 44.37 must be considered a normal figure. The interval between the onset of symptoms and occurrence of death in fatal cases affords the only other criterion of severity available in a field investigation of this kind. Four hundred and eighty-one deaths in 23 villages were distributed as follows:—

TABLE I.

Day of death:—	1st	2nd	3rd	4th	5th	6th and after.	Total.
Number ..	280	146	29	8	5	13	481

It will be seen that 426 out of 481 deaths occurred within two days, the period in most cases being less than 24 hours after the onset of symptoms and this would suggest that the infection was of a virulent nature.

Specimens of stools from 131 cases were examined bacteriologically and in 97 cases *V. cholerae* was isolated; 84 of these were studied. They were distributed as follows:—

TABLE II.

Feb.	March.	April.	May.	June.	July.	Aug.	Sept.	Oct.	Nov.	Dec.
5	2	4	15	23	18	6	2	5	3	1

They were all typical cholera vibrios, gave positive cholera-red reaction and were non-hæmolytic [goat-cells, Greig's (1914) technique]. They fermented saccharose and mannose with formation of acid only and did not ferment arabinose. They would thus fall in Heiberg's group I vibrios (Heiberg, 1935). They were tested against Inaba and Ogawa 'O' sera, raised from Bruce-White's dried standard cholera antigens and were all found to be of the Ogawa type. The following table is a sample of the results obtained and shows the clear differentiation of the types:—

TABLE III.

Vibrio number.	AGGLUTINATION PER CENT TITRE.	
	Inaba 'O'.	Ogawa 'O'.
G. 36135 ..	10	80
G. 36136 ..	10	80
G. 36137 ..	10	100
G. 36138 ..	10	100
G. 36154 ..	10	75
G. 36155 ..	10	75
G. 36156 ..	10	100
G. 36157 ..	10	100
G. 36158 ..	10	100
G. 36159 ..	10	75
G. 36160 ..	10	100
G. 36161 ..	10	100
G. 36162 ..	10	75
G. P. 6 ..	10	100

The homologous 'O' titre of the two sera was 2,500.

For further and final confirmation absorption tests were done with 'O' sera raised from three of these strains, viz., G. 36154, G. 36157, and G. 36158, and the Inaba and Ogawa sera. The results are shown in Table IV and establish the serological identity of these vibrios with the Ogawa type:—

TABLE IV.

Serum culture.	OGAWA 'O'.				INABA 'O'.				G. 36154 'O'.				G. 36157 'O'.				G. 36158 'O'.			
	ABSORBED WITH				Unabsorbed.	ABSORBED WITH				Unabsorbed.	ABSORBED WITH				Unabsorbed.	ABSORBED WITH				
	36154	36157	36158	36154		36157	36158	Inaba.	Ogawa.		Inaba.	Ogawa.	Inaba.	Ogawa.		Inaba.	Ogawa.			
G. 36154	2,500	0	0	0	500	0	0	0	2,500	2,500	0	2,500	2,500	0	2,500	2,500	0	1,250	1,250	0
G. 36157	2,500	0	0	0	500	0	0	0	2,500	2,500	0	2,500	2,500	0	2,500	2,500	0	1,250	1,250	0
G. 36158	2,500	0	0	0	500	0	0	0	2,500	2,500	0	2,500	2,500	0	2,500	2,500	0	1,250	1,250	0
Inaba ..	250	0	0	0	2,500	2,500	2,500	1,250	0	0	0	0	0	0	0	0	0	0	0	0
Ogawa ..	2,500	0	0	0	500	0	0	0	2,500	2,500	0	2,500	2,500	0	2,500	2,500	0	1,250	1,250	0

0 = less than 50 the lowest dilution tested.

24-hour growth from 1 Roux bottle was used to absorb 1 c.c. of serum diluted 1 in 20 : contact at 37°C. for two hours and in ice box overnight. Agglutination tests were left in a water-bath at 52°C. to 54°C., for 18 to 20 hours.

SUMMARY.

A severe epidemic of cholera in Periyakulam range of the Madura district in South India is recorded and the vibrios isolated have been shown to be of the 'Ogawa' type of Gardner and Venkatraman.

ACKNOWLEDGMENT.

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ON THE VALUE OF WILSON AND BLAIR'S BISMUTH
SULPHITE MEDIUM IN THE ISOLATION OF *BACT.*
TYPHOSUM FROM RIVER WATER.

BY

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SANITARIANS in the past recognized the presence of pathogenic organisms (for example, typhoid-paratyphoid group) in sewage and water which gave rise to epidemics and rightly directed their attention to their purification as an essential step for the improvement of public health. The actual demonstration of such organisms in sewage and water proved, however, to be of the utmost technical difficulty. Organisms of typhoid and paratyphoid groups, when present in a communal sewage or water, are so much diluted and outnumbered by other saprophytic organisms that, even with selective methods, very great difficulty was experienced in their isolation. The introduction of Wilson and Blair's (1927) medium made a great advance in this direction and has enabled us to investigate the incidence and viability of these organisms outside the human body with a reasonable degree of precision. In a previous paper (Stewart and Ghosal, 1932) we produced evidence indicating the value of this medium for the isolation of and investigation of the viability of *Bact. typhosum* in sewage and septic tank effluents. In the present communication we present our experience of the same medium in the isolation of this bacillus from the River Hooghly, a large tidal river flowing on the western side of Calcutta.

In 1929 Houston isolated *Bact. typhosum* from the River Thames by Wilson and Blair's medium. Before this, he had failed to do so by all other technique.

Later, Wilson and Blair (1931) succeeded in isolating the organism from the River Lagan and also from a dam in the course of a stream, while Scott, with the same medium, demonstrated the bacillus in the River Var. Using this method several recent outbreaks of enteric fever in England could be traced to contamination of water-supplies by sewage.

TECHNIQUE OF ISOLATION.

One sample was collected each day at a fixed time from a bathing 'ghat' and examined in the laboratory in the following manner:—

To 1,000 c.c. of the sample (which was always alkaline in reaction) was added a 1 per cent solution of acid potassium phosphate till the pH was brought down to 7·0 to 6·8, as this facilitated rapid sedimentation. Two c.c. of a 5 per cent alum solution were then added and, after shaking, it was allowed to settle for a few hours. On settlement, the supernatant fluid was thrown off, the last 200 c.c. were centrifuged and the deposit plated on Wilson and Blair's medium. (The composition of the medium was the same as that described in our previous paper except that no absolute alcohol was added.) The subsequent steps in the isolation and identification of *Bact. typhosum* were those described in our last paper.

RESULTS.

Seventy-eight samples were examined from January 1935 to February 1936 of which eight yielded *Bact. typhosum*. The positive findings may be divided into two groups:—

GROUP I.—This group consists of five samples examined in the first week of February 1935 during 'Ardhodaya Yoga' which is a great Hindu festival, at which hundreds of thousands of pilgrims visit Calcutta and take a bath of purification in the River Hooghly. Of the five samples three gave positive results. Such a high percentage of positive findings in one week was not found on any other occasion. As a control three more weekly examinations of five samples were carried out in different months with only one positive result.

GROUP II.—This comprises the other five positive findings obtained from the remaining samples on different occasions.

It may be mentioned here that great difficulty was experienced during the monsoon months in isolating *Bact. typhosum*. Plates were overcrowded with the black colonies of other organisms and the isolation of *Bact. typhosum* was a hopeless task. Thirteen samples were examined during the monsoon and if these are excluded the results showed eight positives among the 65 samples examined.

DISCUSSION.

The results clearly demonstrate the value of Wilson and Blair's medium in the isolation of *Bact. typhosum* from river water. The isolation of the typhoid bacillus from such a large river as the Hooghly was previously not considered to be a practicable proposition. The findings are also of considerable interest from the epidemiological point of view. Enteric fever is endemic in Calcutta and it is well known that a large section of the inhabitants are in the habit of bathing in the

river, of drinking its water, and of washing their utensils with the river water. Though the local health authorities had drawn attention previously to the danger of river water in the spread of enteric fever, the actual demonstration of the organism in the water had been wanting and hence the warnings may have failed to carry conviction. With the isolation of the bacillus this link has been found, and the public will probably now be more convinced of the dangers attendant on the use of Hooghly water. The increase in the positive findings during the 'Ardhodaya Yoga' festival suggests that the bacilli were coming from human carriers.

SUMMARY.

(1) *Bact. typhosum* was isolated eight times from the River Hooghly out of 78 samples examined and, excluding the samples examined during the monsoon, the results were eight positives out of 65 samples examined.

(2) The number of positive findings increased at the time of the 'Ardhodaya Yoga' festival, thus suggesting that the bacilli were being derived from human sources.

(3) The risk of using river water for bathing, drinking, and domestic purposes has been emphasized.

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RESPONSE OF THE CHORIO-ALLANTOIC MEMBRANE OF
THE DEVELOPING CHICK-EMBRYO TO INOCULATION
WITH VARIOUS SUBSTANCES WITH
SPECIAL REFERENCE TO
B. PROTEUS X 19.

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THE chorio-allantoic membrane of the developing egg has been used extensively in the cultivation of filterable viruses. The interpretation of the results obtained in the membranes usually presents no difficulty in the case of known viruses as, in the majority of these, susceptible laboratory animals are available to test the lesion material. It is when one seeks to establish the virus nature of a disease by attempting to obtain primary cultures in egg membranes from inoculation of material supposed to contain the virus, that considerable caution is needed in the interpretation of the results obtained, especially if the hypothetical virus is not readily transmissible to laboratory animals. We have experienced this difficulty while attempting to cultivate the trachoma virus (Pandit, Wright and Sanjiva Rao, 1935) and sandfly fever virus (Shortt, Rao and Swaminath, 1936) in egg membranes. The lesions obtained in these instances were transmissible in series from egg to egg after filtration through suitable graded membranes. This finding suggested the probability that the lesions obtained were due to viruses and were not merely the result of physical and mechanical injuries which are known to produce lesions not greatly dissimilar to those usually noted in known virus infections. Owing, however, to the difficulties inherent in interpreting the response of the chorio-allantoic membrane to differing stimuli it was considered essential to study these responses to various substances definitely known not to contain any virus. This investigation is still in progress, but certain facts have emerged which we consider are of sufficient interest to be recorded.

MATERIALS AND METHODS.

The substances employed belong to various categories. These were grouped as follows:—

1. *Insoluble substances* such as Kieselguhr 1 per cent suspension, aluminium gel 5 per cent suspension, Indian ink 1 per cent suspension, and olive oil.
2. *Soluble substances* such as copper sulphate 1 per cent solution, normal saline, glycerine 50 per cent solution, and starch 1 per cent solution.
3. *Biological substances* such as ground normal membrane, normal and febrile human serum, milk, sterile bacterial filtrates, ptyalin, and pepsin.
4. The following bacteria: *E. typhosus*, *B. proteus*, *V. cholerae*, *C. diphtheriae*, *N. catarrhalis*, *Staphylococcus aureus*, and *Streptococcus non-haemolyticus*.

Eggs 13 to 14 days old were used for inoculation, as they were found to be more suitable than younger eggs. The technique of inoculation was the same as that described by Rao, Pandit and Shortt (1936) in their study of vaccinia, each side of the triangular opening being about half an inch long. The amount of inoculum in each case was 0.15 c.c. The soluble and insoluble substances were all sterilized by heat, and the biological substances were tested for sterility prior to inoculation. The bacterial suspensions were standardized to contain 500 million organisms per c.c. as higher strengths of some organisms were fatal to embryos.

The inoculated eggs were incubated at 39°C. and opened on the fourth day. Our attention was confined to the membranes, and the embryos were not investigated except in the study of *B. proteus*. In the case of bacteria a longer incubation period led to a high mortality among the embryos. In all cases the lesions, as well as the sites of inoculation not showing naked-eye lesions, were examined in sections. The lesions, when ground, were tested bacteriologically for purity and sterility, as the case might be, prior to further passages in eggs.

RESULTS.

It was found that aluminium gel alone, of inert substances, and bacteria produced lesions not unlike those of virus infections. It may be noted here that all marginal lesions, opacities, or thickenings of the inoculated areas usually met with as responses to mechanical injuries were not considered as lesions. A lesion was only recorded as such when there was a distinct heaping up at the centre and slope towards the periphery, and when such a lesion occupied the centre or a greater portion of the area inoculated. This standard was adopted as it was our intention to assess only such responses as are usually noted in virus infections. The lesions noted had usually a whitish appearance with a slight yellow tint and hæmorrhagic areas around them were not noted.

Before describing the lesions produced by the various agents employed in our experiments the appearances seen in a lesion, the result of mechanical injury, may be briefly described for purposes of comparison. The section of such a lesion shows considerable proliferation of the ectoderm. The entoderm is little affected if, in the specimen studied, the injury has been very superficial. The mesoderm

is thickened with fibroblastic infiltration and the presence of a certain number of eosinophiles is noted. Some oedema of the mesoderm is seen and cell nests are present.

LESIONS PRODUCED BY ALUMINIUM GEL.

The aluminium gel was prepared as follows: 147 grammes of aluminium sulphate, equivalent to 22.5 grammes of Al_2O_3 , were dissolved in 300 c.c. of distilled water, and strong ammonia (0.88 sp. gr.) was added slowly until the precipitate obtained had a strong persistent smell of ammonia. The precipitate was then diluted to three times its bulk with tap-water, and allowed to settle. The supernatant liquid was drawn off. This operation was repeated two or three times until there was very little smell of ammonia. Two hundred c.c. of distilled water were added to the precipitate, and the suspension boiled until there was no trace of ammonia. The precipitate was finally washed with distilled water, and allowed to settle. The supernatant liquid was drawn off as completely as possible. The volume of the precipitate was measured and made up to one litre with ammonia-free distilled water. From this a 5 per cent suspension was made in distilled water, sterilized by boiling, and used for inoculation.

The naked-eye appearance of the lesions obtained was as noted above. No focal lesions were found in any of the membranes.

Microscopic appearances.—Sections show considerable proliferation of the ectoderm with a tendency to papillation, a much slighter proliferation of the entoderm, and marked thickening of the mesoderm with cellular infiltration with fibroblasts. Eosinophiles are also present and there are large irregular masses of structureless homogeneous eosinophilic-staining substance. Cell nests are present and the lesions are markedly vascular.

The lesions were ground with papain broth, about 2.5 c.c. being used for one lesion. They were found to be sterile when cultured on the routine bacteriological media. The inoculation of ground material, as well as the filtrate obtained after filtering it through a gradocol membrane of 0.9μ average pore diameter, did not give rise to any lesion.

LESIONS DUE TO BACTERIA.

The species of bacteria investigated have already been listed. As has been noted by other workers, lesions were obtained with all of them on primary inoculation. The naked-eye characters of these lesions agreed with the general description already given. Discrete or focal lesions, in addition to the main lesions, were also noted, particularly with *B. proteus*. Hæmorrhagic areas around the lesions were never found. The lesions obtained with bacteria are dealt with below in more detail.

Lesions with B. proteus.—The lesions were obtained with the three strains used, viz., *B. proteus* X 19, X 2, and X K.

Microscopic appearances.—Sections show proliferation of both ectoderm and entoderm, especially the former. The mesoderm is greatly thickened, with oedema and marked fibroblastic infiltration with some eosinophiles. Cell nests are present.

From the lesion the organism inoculated could be isolated in pure culture. As has been noted by Burnet (1936) great variability was noted in the production of lesions in the batch of eggs inoculated with the same inoculum. From the heart

blood of living embryos the organisms were isolated in pure culture, irrespective of whether lesions had appeared on the membrane or not. The cultures isolated showed no change in their agglutinability after six serial passages in egg membranes. Biochemical reactions were also constant, except on one occasion in each case with *proteus* X 19 when gas production was inhibited, with *proteus* X 2 when maltose was not fermented, and with *proteus* X K when maltose, saccharose, and salicin were not fermented. No change in the antigenic characters of *proteus* X 19 was noticeable, when this strain was tested with homologous antiserum by the agglutination absorption method.

Other strains were not investigated from this point of view.

Filtrability of lesions produced by proteus X 19.—The lesions obtained with this strain were ground in papain broth and filtered through Elford's gradocol membranes of 0.9μ average pore diameter. The filtrate was found to be sterile when tested on all routine laboratory media, after both aerobic and anaerobic cultivation, and after incubation for a period of 7 to 10 days. The sterile filtrates inoculated into eggs again produced well-marked lesions. It was found that these lesions, although bacteriologically sterile, could be passaged in series, apparently indefinitely, even after intermediate filtration. If the ground lesion suspension was centrifugalized, lesions were produced both with the clear supernatant, and with the deposit, though the lesions with the latter tended to be more pronounced. Actually 21 serial passages were carried out. The details are given in Table I:—

TABLE I.

Number of passage.	Number of eggs inoculated.	Number of eggs showing lesions.	Degree of lesions.
1	3	1	+++
2	4	2	++++
3	4	3	+
4	11	7	++
5	6	4	++
6	6	3	+++
7	8	5	++++
8	10	8	+++
9	7	7	++
10	9	6	+++
11	10	7	++++
12	12	4	++
13	8	6	+++
14	8	3	+
15	9	5	++++
16	8	3	+++
17	9	5	+++
18	10	4	+++
19	9	4	++
20	4	2	+
21	3	2	+++

++++ = Lesion which occupies the whole of the inoculated area.

++ = Small circumscribed lesion in the centre of the inoculated area.

++ and +++ = Lesions intermediate in size.

It will be seen from this table that the response in the eggs was not uniform and great variability in the size of lesions was noticed in any one of a batch of eggs inoculated with the same inoculum.

When the emulsions of lesions were filtered through gradocol membranes of 0.6μ average pore diameter, no lesions resulted in the inoculated eggs. It would appear, therefore, that the agent in the sterile filtrates causing lesions in egg membranes is of a particulate nature.

It might also be stated that when the filtrate lesion suspensions were heated to 100°C . no lesions resulted on their subsequent inoculation into eggs. Heating to 60°C . for 30 minutes did not affect the production of lesions.

Attempts to cultivate the original organism from the filtrate lesions on the routine laboratory media have proved so far entirely negative. During the course of this investigation, however, a Gram-negative motile bacillus was occasionally grown in pure culture, which suggested the possibility of its being a modified form of *B. proteus* X 19. This organism fermented after 48 to 72 hours glucose, maltose, and saccharose without gas production. Litmus milk was made alkaline. It was not agglutinated by rabbit antisera against *proteus* X 19, X 2, or X K. Subsequent examinations revealed that this organism was a normal or occasional commensal present in the egg embryos. It was isolated both from the membrane and heart blood of living chick-embryos from uninoculated eggs.

Microscopic appearances of filtrate lesions.—The sections show marked proliferation of both ectoderm and entoderm, mesoderm much thickened, with marked fibroblastic infiltration and presence of cell nests. The lesion is very vascular and with marked eosinophilic response, the section in some cases being crowded with eosinophil cells. The fibroblasts below both ectoderm and entoderm are arranged parallel with the surface producing a sort of 'felted' effect.

Lesions with E. typhosus.—Results of inoculation with *E. typhosus* were identical with those obtained with *B. proteus* X 19. Thus the lesions could be passaged in series in eggs after filtration of the ground lesion suspension through gradocol membranes of 0.9μ average pore diameter. Only three serial passages were carried out. Heating to 100°C . destroyed the agent responsible for the production of lesions, while heating to 60°C . for 30 minutes did not affect the power of producing lesions.

Microscopically *E. typhosus* lesions show proliferation of both ectoderm and entoderm, especially the former. There is thickening of the mesoderm with fibroblastic infiltration and the presence of cell nests. Eosinophiles are also present. *E. typhosus* filtrate lesions show the same picture without the presence of cell nests.

Lesions with other bacteria.—The primary lesions with *C. diphtheriae*, *Staphylococcus aureus*, and *Streptococcus non-haemolyticus* were not found capable of propagation in series after filtration. With *V. cholerae* and *N. catarrhalis*, however, some evidence was obtained which indicated the presence of a filter-passing and lesion-producing agent in primary lesions. This question has not been studied in detail.

Microscopic appearances of lesions with V. cholerae.—The sections show marked proliferation of the ectoderm, less marked of the entoderm. The mesoderm is

greatly thickened, showing fibroblastic infiltration, a few eosinophiles, and cell nests. Marked œdema is also present.

Microscopic appearances of lesions with Staphylococcus aureus.—The section shows very marked proliferation of ectoderm as well as marked proliferation of entoderm. The mesoderm is much thickened with fibroblastic infiltration. It shows marked œdema and a great development of cell nests. Eosinophiles are also present.

The results obtained with bacteria are summarized in Table II:—

TABLE II.

Inoculum.	Nature of lesions in eggs.	Average pore diameter of gradocol membrane.	Result of filtrate inoculations.	Number of serial passages with filtrate lesion.	REMARKS.
<i>B. proteus</i> X 19 ..	++++	0.9 μ	++++	21	No lesions when filtered through 0.6 μ membrane. Heating ground material to 100°C. = No lesion, to 60°C. for 30 minutes = Lesions.
<i>B. proteus</i> X 2 ..	+++	} Not done			
<i>B. proteus</i> X K ..	+++				
<i>E. typhosus</i> ..	+++	0.9 μ	+++	3	
<i>V. cholerae</i> ..	+++	0.9 μ		..	} Results inconclusive.
<i>N. catarrhalis</i> ..	++++	1.3 μ		..	
<i>Staphylococcus aureus</i>	++++	1.1 μ	Negative	..	
<i>C. diphtheriae</i> ..	+++	1.3 μ	Negative	..	
<i>Streptococcus non-hæmolyticus</i> .	+ ?	1.1 μ	Negative	..	Lesions not typical. Membrane thickened and opaque.

Inoculated organisms were isolated in pure culture from all primary lesions. The filtrates and filtrate lesions in serial passages remained sterile on routine bacteriological media.

COMMENTS.

Lesions of the type we have described were not observed with any of the inert substances studied, with the exception of aluminium gel. Histologically, also, there was no evidence of any definite lesion having been produced at the inoculated sites. D'Aunoy and Evans (1937) have reported the presence of lesions with histological changes, similar to those recorded by us in the case of aluminium gel and bacteria, even in normal uninoculated membranes. The authors do not mention, however, whether such membranes were examined bacteriologically and found to be sterile. For, as we have shown, from membranes and living embryos from uninoculated eggs a Gram-negative bacillus could be isolated which, when inoculated into eggs, produced lesions. Even so this organism was only met with occasionally, while D'Aunoy and Evans reported the lesions quite regularly in normal membranes.

No explanation can be offered by us as to why aluminium gel alone of the inert substances produced lesions in eggs. This finding is, however, of some value in that such lesion material could be used with advantage as control material in the study of viruses present in membrane lesions.

The results obtained with *B. proteus* and *E. typhosus* appear to us capable of one obvious explanation, viz., the agent in sterile filtrates causing lesions might be a filterable form of these organisms. It would be interesting to note in this connection that Chapman and McKee (1935) have noted a filterable form of *B. proteus* X 19 and *E. typhosus* when grown in *K* medium.

Another explanation might be that the filterable lesions were due to a proteolytic enzyme produced initially by *B. proteus* in the membrane and subsequently carried through from membrane to membrane even after filtration. This possibility was investigated by the digestion of carmine-stained fibrin by filtrate lesion material. Normal membrane material was used as a control. Apart from the obvious limitations of this method of testing the enzyme action in lesions the experiments proved indecisive although evidence of any appreciable amount of ferment was lacking.

An examination of the lesions produced by the various agents used in the study gives us the impression of a very uniform histology whatever the agent producing the lesion may be. Thus, in all cases, there is more or less marked proliferation of ectoderm and entoderm and a thickening of mesoderm with infiltration of fibroblastic cells. The only obvious deviation from this general picture was in the case of lesions produced by filtrates of *B. proteus* X 19 lesions in which there was a very marked eosinophilic response similar to that known to be an accompaniment of the response of the chorio-allantoic membrane to some virus infections.

SUMMARY.

1. The response of the chorio-allantoic membrane to inoculation with substances known definitely not to contain any virus was studied. The substances used included soluble and insoluble substances, biological substances, and bacteria. It was found that aluminium gel alone, of the inert substances, and bacteria produced lesions in eggs not dissimilar to those met with in known virus infections.

2. The lesions produced by *B. proteus* X 19 and *E. typhosus* were capable of being propagated in series after filtration of the ground lesions through gradocol membranes.

3. From bacterial lesions the organisms inoculated were always isolated in pure culture. The filtrate lesions in all serial passages made remained sterile on routine laboratory media.

4. Microscopically the lesions had a more or less uniform histology whatever the agent producing the lesion might be. In lesions produced by the filtrates of *B. proteus* X 19 lesions, however, the eosinophilic response was more marked than that seen in sections of primary lesions produced by that organism.

5. Attention is drawn to the presence of a motile Gram-negative bacillus in the membranes and heart blood of a few living chick-embryos from uninoculated eggs.

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LABORATORY TESTS ON THE FUNGISTATIC AND FUNGICIDAL EFFECT OF VARIOUS SUBSTANCES.

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NOTWITHSTANDING the large amount of laboratory work that has been done in testing drugs and chemicals of many kinds for their action on fungi, with the object of finding a reliable cure for the various fungus infections of man and animals, no entirely satisfactory ones are yet available. Another disappointing fact is that, in our experience, a preparation strongly recommended elsewhere may be found relatively useless when tried on apparently the same disease under Indian conditions. This makes it imperative to try promising remedies on local strains of the fungi before a definite opinion on their value can be given for India. We have accordingly carried out tests on a number of preparations and give in this paper the results so far achieved; it is proposed to detail our clinical results, based on the *in vitro* findings given here, in a second paper.

In selecting the drugs deemed worthy of detailed study, from among the immense numbers which are possibly of use in this direction, we have to some extent been guided by other workers in the same field, notably Schamberg and Kolmer (1931) and Kingery and Adkinsson (1928), and we have also tried preparations sent with recommendations by the manufacturers or that have been reported as useful in other places, on clinical results alone.

Epidermophyton cruris and the several allied or, as some consider, the identical species, is by far the commonest type of infection, so this species has naturally been very extensively used as the standard for testing fungicidal compounds, but

infections with the trichophyton group are much more difficult to cure by local applications so we have also paid a good deal of attention to them in this inquiry. One species of special interest to us is *Achorion violaceum* because it is fairly prevalent and infection with it is nearly always complicated by a staphylococcal infection of the follicles as well, which makes the cure much more difficult. Local strains of four common fungi were used throughout the inquiry and the same strain of each was used the whole time. They were: *Epidermophyton cruris*, *Achorion violaceum*, *Achorion actoni*, and *Microsporum audouini*.

TECHNIQUE.

Our methods are based on those of Schamberg and Kolmer (1922) but several modifications to suit our special needs were introduced. Our test fungi were invariably grown on Sabouraud's maltose peptone agar, with a pH of 6.0, for a period of three to four weeks, because this is the time that the fungi are at their greatest activity and are furnished with their characteristic end-organs. The complete* surface growth was carefully removed in small pieces, avoiding as far as possible inclusion of any of the medium. The growth was placed in a sterile bottle containing some sterile glass beads and 10 c.c. of sterile distilled water added; after this the bottle was shaken mechanically for about three hours, by which time a fine uniform emulsion had formed. The emulsions so made were always very opaque so a sample was diluted about ten times further with distilled water after which it could be matched against a set of Wright's standard opacity tubes and it was finally adjusted to an opacity of between 900 and 1,000 millions of organisms per c.c. Measured quantities of such an emulsion thus had approximately the same numbers of organisms.

Two sets of tests were made with each drug, the fungistatic and the fungicidal, because preliminary fungistatic tests reduced the number necessary to ascertain the highest fungicidal solution of a drug.

Fungistatic test.—Sabouraud's maltose peptone agar (pH 6.0) was used but of course the hydrogen-ion concentration would be altered by the addition of the different preparations to be tested, and the pH of the medium plus the test substance were not taken. Nine c.c. of culture medium were liquefied in a water-bath and 1 c.c. of the test substance in solution thoroughly mixed with it, thus giving a dilution one-tenth the strength of the original. So as to obviate possible decomposition of the test substance by heat the liquefied medium was allowed to cool to as low a temperature as would allow of the test solution mixing thoroughly with it. This was found to be 45°C.; at lower temperatures the medium became too solid to allow of even diffusion through it of the test solution.

Ordinary slopes in test-tubes were made with the mixtures so prepared under strict aseptic precautions so that subsequent sterilization, with probable inactivation of the test substance, would not be necessary. In the fungistatic tests inoculations were made with small portions of unemulsified stock culture, and the results were read twenty-one days later.

* In the case of *A. violaceum* it was necessary to take the contents of three or four culture tubes to obtain sufficient material.

Fungicidal tests.—One half c.c. of standard emulsion, prepared as described above, was placed in a test-tube, four inches long by half an inch in diameter, and an equal quantity of the test solution was added, using sterile graduated pipettes for the purpose. It should be noted that the dilution of the test substance was doubled by this operation. The mixture was shaken about sixty times in a period of half a minute and placed aside until needed. Every drug at each dilution used was tested by taking loops of the appropriate mixtures at intervals of 1 minute, 10 minutes, 30 minutes, 1 hour, and 24 hours and inoculating them on Sabouraud's agar slopes. The mixtures were well shaken each time before a loop was taken for inoculation. The inoculated tubes were examined daily for fifteen days and if by that time no growth was detected the fungus was considered to have been killed. Each test was controlled by a plant of simple emulsion from the same stock as those from which the test mixtures were made.

Because of our experience of the frequent complication of *A. violaceum* by a pustular folliculitis a strain of *Staphylococcus aureus* isolated from a case with this condition was also tested against many of the same drugs, with the object of finding the best one for curing both the bacterial and fungal infections at the same time, or possibly of combining the two drugs shown to be the best against the two organisms.

DISCUSSION OF RESULTS.

Reference to the tables will show our results and they need little discussion for they are for the most part self-explanatory.

The substances have been arranged in the tables in the order of their efficiency as far as possible, the best drug being at the bottom, thus giving a graphic representation of their powers. When the objects of this research are recalled this classification seems preferable to grouping drugs of similar chemical composition and properties regardless of their action on fungi.

Although for the sake of clarity only the lowest dilution which failed to prevent growth is indicated by a plus sign in the tables, tests above and below these dilutions were of course carried out, and although only one result is given it was confirmed by identical tests on more than one occasion in every instance. In some cases the gap between the last negative and the first positive dilutions is rather wide, and work is now in progress to try intermediate dilutions in these cases so as to define more accurately the end-point of action of the substances.

Abracide is the proprietary name for a liquid containing 10 per cent of hydroxymethyl-butyl-benzene, which is structurally similar to thymol. It will be seen that the fungistatic action of abracide and thymol is identical, but as a fungicide the former is slightly better.

Cinnamon oil was found to be a better fungistatic agent than thymol, but its fungicidal action was not good. It is an unsatisfactory substance because different samples of oil showed very different powers.

Lugol's solution suitably diluted was the source of the iodine used.

Paranitrophenol came to us with glowing accounts of its effect on ringworm of the feet in the Malay States. It had been used empirically by laymen

because it is used commercially to prevent moulds growing on raw rubber during shipment.

Benzoic acid is only soluble in water 1/450 and salicylic acid 1/500 so for practical purposes the strongest effective solution it is possible to make in both may be taken as 1/500. In our technique 1 c.c. of the solution was added to 9 c.c. of culture medium giving a constant proportion of water and medium in all tests. Under these conditions, and to maintain uniformity in all our tests, 1/5,000 was the highest concentration in which these two acids could be tested in the fungistatic tests. They do not seem very efficient as fungicides although they are well-known remedies, especially in Whitfield's ointment where benzoic acid is present in 5 per cent strength and salicylic acid in 3 per cent.

It will be seen that brilliant green is much more effective against *A. violaceum* than crystal violet, but crystal violet is better against the staphylococcus we used than is brilliant green, so a mixture of these two dyes was made. The action of the combined dyes was disappointing in their effect on *A. violaceum*, the reason of making the combination, but it was surprisingly effective against *E. cruris*. It is possible a new compound is formed in the mixture.

We give below the origin of the various substances used in this research, not with the object either of recommending or condemning any special preparations used, but as an indication of their quality so that other workers may be able more easily to assess our results.

The benzoic and salicylic acids, mercuric chloride, iodine, thymol, clove and cinnamon oils, acriflavine, and quinine were all of British Pharmacopœia standard; the pure paranitrophenol and gentian violet were manufactured by Merck; the fuchsin, crystal violet, malachite green, and brilliant green were from Grubler's factory; the mercurochrome and merfenil were supplied by May and Baker; the abraicide by A. Boake, Robert & Co., and the commercial paranitrophenol was the same as is used in the preservation of raw rubber.

SUMMARY.

The *in vitro* effect of a collection of substances against four strains of common fungi is given and the technique employed is described in detail.

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TABLE I.
Fungistatic tests.

Dilution :—	<i>Epidermophyton cruris.</i>										<i>Asclerion violaceum.</i>									
	1/1,000.	1/2,000.	1/4,000.	1/5,000.	1/10,000.	1/20,000.	1/40,000.	1/60,000.	1/100,000.	1/1,000,000.	1/1,000.	1/2,000.	1/4,000.	1/5,000.	1/10,000.	1/20,000.	1/40,000.	1/100,000.	1/200,000.	1/400,000.
Quinine bishydrochlor.	+									+										
Iodine ..																				
Paranitrophenol (pure) ..		+																		
Acid benzoic																				
Acid salicylic																				
Fuchsin (basic)																				
Hydrag. perchlor.																				
Mercuræobrom																				
Gentian violet																				
Thymol ..																				
Abracide ..																				
Clove oil ..																				
Crystal violet																				
Cinnamon oil																				
Acridine ..																				
Brilliant green																				
Malachite green																				
Brilliant green																				
Crystal violet																				
Mercuril ..																				

+ = growth.

- = no growth.

Blank = test not done.

All the dilutions shown in the table were used for each species of fungus, but when there was no growth with any of the drugs used in any given dilution it has been omitted from the table under the species concerned.

TABLE I—*concl.*

Dilution :—	<i>Microsporium audouinii.</i>										<i>Achorion actoni.</i>									
	1/1,000.	1/2,000.	1/4,000.	1/5,000.	1/10,000.	1/20,000.	1/40,000.	1/50,000.	1/100,000.	1/160,000.	1/200,000.	1/400,000.	1/600,000.	1/800,000.						
Quinine bilhydrochlor.	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Iodine	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Paranitrophenol (pure)	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Acid benzoic	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Acid salicylic	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Fuchsin (basic)	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Hydraz. perchlor.	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Mercurochrome	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Gentian violet	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Thymol	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Abacide	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Clove oil	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Crystal violet	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Cinnamon oil	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Acriflavine	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Brilliant green	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Malachite green	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Brilliant green	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Crystal violet	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Merfenil	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:

+ = growth.

— = no growth.

Blank = test not done.

All the dilutions shown in the table were used for each species of fungus, but when there was no growth with any of the drugs used in any given dilution it has been omitted from the table under the species concerned.

TABLE II.

Bacteriostatic results with a strain of Staphylococcus aureus isolated from a case of A. violaceum infection.

Dilution :—		1/1,000.	1/5,000.	1/10,000.	1/20,000.	1/40,000.	1/80,000.	1/160,000.	1/2,000,000.
Quinine bihydrochlor.	+							
Acid benzoic	-	+						
Acid salicylic	-	+						
Iodine	-	-	+					
Clove oil	-	-	+					
Cinnamon oil	-	-	+					
Acridavine	-	-	+					
Fuchsin (basic)	-	-	-	+				
Brilliant green	-	-	-	+				
Mercurochrome	-	-	-	+				
Malachite green	-	-	-	-	+			
Hydrarg. perchlor.	-	-	-	-	-	+		
Crystal violet	-	-	-	-	-	-	+	
Brilliant green }	-	-	-	-	-	-	+	
Crystal violet }									
Merfenil	-	-	-	-	-	-	-	+

Many more dilutions were tried than are shown in the table, but only those in which a growth occurred with one or more drugs are given.

TABLE III.

*Fungicidal tests.**Epidermophyton cruris.*

Time :—	1 Minute.										10 Minutes.								30 Minutes.										
	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/12,000.	1/14,000.	1/50,000.	1/60,000.	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/7,000.	1/14,000.	1/100,000.	1/200,000.	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/10,000.	1/14,000.	1/200,000.
Dilution :—																													
Quinine bitydrochlor.	+																												
Paranitrophenol (pure)	+																												
Paranitrophenol (conml.)	+																												
Acid benzoic ..	+																												
Clove oil ..	+																												
Cinnamon oil ..	+																												
Fuchsin (basic)	+																												
Thymol ..	+																												
Mercurochrome ..	+																												
Abracide ..	+																												
Acid salicylic ..	+																												
Gentian violet ..	+																												
Crystal violet ..	+																												
Acridavine ..	+																												
Hydrarg. perchlor.	+																												
Malachite green ..	+																												
Iodine ..	+																												
Brilliant green ..	+																												
Merfenil ..	+																												
Brilliant green } Crystal violet }	+																												

See notes under Table 1.

TABLE III—*concl'd.*

TIME :—	1 Hour.										24 Hours.													
	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/8,000.	1/9,000.	1/10,000.	1/14,000.	1/200,000.	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/8,000.	1/9,000.	1/10,000.	1/14,000.	1/200,000.
Dilution :—																								
Quinine bilydrochlor.	+																							
Paranitrophenol (pure)	+																							
Paranitrophenol (comm.)	+																							
Acid benzoic ..	+																							
Clove oil ..	+																							
Cinnamon oil ..	+																							
Fuchsin (basic) ..	+																							
Thymol ..	+																							
Mercurochrome ..	+																							
Abracide ..	+																							
Acid salicylic ..	+																							
Gentian violet ..	+																							
Crystal violet ..	+																							
Acridflavine ..	+																							
Hydrag. perchlor.	+																							
Malachite green ..	+																							
Iodine ..	+																							
Brilliant green ..	+																							
Merfenil ..	+																							
Brilliant green } Crystal violet }	+																							

See notes under Table I.

TABLE IV.
Fungicidal tests.
Achorion violaceum.

Dilution :—	1 Minute.									10 Minutes.									30 Minutes.														
	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/10,000.	1/20,000.	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/7,000.	1/8,000.	1/9,000.	1/10,000.	1/14,000.	1/20,000.	1/60,000.	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/7,000.	1/8,000.	1/9,000.	1/10,000.	1/14,000.	1/20,000.	1/60,000.
Paranitrophenol (pure) ..	+									+												+	+	+	+	+	+	+	+	+	+	+	+
Paranitrophenol (comm.)	+									+																							
Acid benzoic ..	+									+																							
Clove oil ..	+									+																							
Cinnamon oil ..	+									+																							
Fuchsin (basic) ..	+									+																							
Malachite green ..	+									+																							
Acridavine ..	+									+																							
Mercurochrome ..	+									+																							
Acid salicylic ..	+									+																							
Brilliant green }	+									+																							
Crystal violet }	+									+																							
Gentian violet	+									+																							
Crystal violet	+									+																							
Thymol ..	+									+																							
Abracide ..	+									+																							
Merfenil ..	+									+																							
Brilliant green	+									+																							
Iodine ..	+									+																							

See notes under Table I.

TABLE IV—concl'd.

TIME :—	1 Hour.												24 Hours.											
	1/1,000.	1/2,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/8,000.	1/9,000.	1/10,000.	1/14,000.	1/16,000.	1/20,000.	1/60,000.										
Dilution :—	Parantrophol (pure) ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Parantrophol (comm.)	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Acid benzoic ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Clove oil ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Cinnamon oil ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Fuchsin (basic)	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Malachite green	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Acridine ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Mercurochrome	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Acid salicylic ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Brilliant green } ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Crystal violet	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Gentian violet	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Crystal violet ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Thymol ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Abracide ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Merfenil ..	+	+	+	+	+	+	+	+	+	+	+	+	+										
	Brilliant green	+	+	+	+	+	+	+	+	+	+	+	+	+										
Iodine ..	+	+	+	+	+	+	+	+	+	+	+	+	+											

See notes under Table I.

Fungicidal tests.

24 Hours.

[illegible]

Acherion actoni.

Time:—	1 Minute.						10 Minutes.						30 Minutes.						1 Hour.						24 Hours.					
	1/1,000.	1/3,000.	1/4,000.	1/8,000.	1/14,000.	+	1/1,000.	1/3,000.	1/4,000.	1/5,000.	1/6,000.	1/7,000.	1/10,000.	1/14,000.	1/1,000.	1/3,000.	1/4,000.	1/5,000.	1/7,000.	1/8,000.	1/10,000.	1/14,000.	1/2,000.	1/4,000.	1/5,000.	1/7,000.	1/8,000.	1/10,000.	1/14,000.	1/1,000.
Dilution:—																														
Paranitrophenol (pure).	+																													
Paranitrophenol (comm.).	+																													
Fuchsin (basic)	+																													
Thymol	+																													
Gentian violet	+																													
Abractide	+																													
Crystal violet	+																													
Brilliant green	+																													

See notes under Table I.

THE IMMUNIZATION OF HORSES FOR THE PRODUCTION OF HIGH-TITRE TETANUS ANTITOXIN.

BY

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THAT the successful production of antitoxin depends on a number of factors, such as the responsiveness of the horse, the quality of the toxin or anatoxin, and the size and spacing of its doses, is well known. When the horses are immunized by the injection of increasing doses of toxin, the antitoxic titre of the serum at first increases, reaches a maximum and then, after a short period, begins to fall. Many attempts have been made to raise the titre of the serum still higher by adding to the antigen, before injection, non-specific substances, such as alum, tapioca, CaCl_2 , MnCl_2 , etc. Thus Ramon and Lemétayer (1932) have shown that the production of diphtheria antitoxin may be improved by injecting to the horses, at first a mixture of tapioca and antigen for some months, and then a mixture of CaCl_2 and antigen. They also report that good results have been obtained, by using this method, in the production of tetanus antitoxin, although in this latter case no actual figures have been given. In this paper we shall record the results of our observations on the immunization of horses for the production of tetanus antitoxin. Our method differs from that of Ramon and Lemétayer in this respect that a brief period of respite was allowed by us to the horses during the course of immunization.

EXPERIMENT.

In our experiments, 27 horses were selected and were first immunized with tetanus toxoid only. They were then divided into three groups. After a brief period of rest the immunization of the horses belonging to the different groups was started again. The horses in group I were injected with increasing doses of antigen only, those in group II were given a mixture of alum and antigen in increasing

doses, while the horses belonging to group III were injected with a mixture of tapioca and antigen. After following this procedure for about two months the horses belonging to group III were subdivided into two groups, viz., group III-A and group III-B. These horses were again given a short period of rest, after which the horses of group III-A received a mixture of CaCl_2 and antigen and those of group III-B received a mixture of MnCl_2 and antigen in progressively higher doses. The results are recorded in Tables I to IV. It will be noticed that horses belonging to group III-A responded very markedly. The average titre of this group rose to 1,490 I. U., while those of the other groups varied from 390 I. U. to 470 I. U.

TABLE I.

Titre of the horses treated with plain antigen.

Horse number.	TITRE IN INTERNATIONAL UNITS IN THE MONTHS OF :—			
	November 1936.	February 1937.	April 1937.	August 1937.
205	0.5	300	500	800
324	0.0	50	200	200
395	0.0	500	500	400
455	0.0	300	200	300
492	0.0	500	400	500
216 (died in May).	5.5	750	1,000	..
504	0.0	100	100	200
484	0.0	50	100	300
AVERAGE ..				390

TABLE II.

Titre of horses treated with alum and antigen.

Horse number.	TITRE IN INTERNATIONAL UNITS IN THE MONTHS OF :—			
	November 1936.	February 1937.	April 1937.	August 1937.
241	0.5	400	500	600
496	0.0	200	400	500
497	0.0	150	400	600
495	0.0	100	300	300
543	0.0	150	200	400
187	0.0	200	400	400
286	0.0	500	400	500
AVERAGE ..				470

TABLE III.

*Titre of horses treated with antigen to which tapioca was added
in the months of March and April and CaCl₂
in the subsequent months.*

Horse number.	TITRE IN INTERNATIONAL UNITS IN THE MONTHS OF :—			
	November 1936.	February 1937.	April 1937.	August 1937.
145	1.0	100	100	1,000
463	0.0	400	600	1,200
445	0.0	300	500	2,000
448	0.0	400	400	3,200
478	0.0	400	400	750
540	0.0	150	300	800
AVERAGE ..				1,490

TABLE IV.

*Titre of horses treated with antigen to which tapioca was added
in the months of March and April and $MnCl_2$
in the subsequent months.*

Horse number.	TITRE IN INTERNATIONAL UNITS IN THE MONTHS OF :—			
	November 1936.	February 1937.	April 1937.	August 1937.
539	0.0	200	500	500
163	0.0	150	400	800
499	0.0	300	500	300
451	0.0	300	400	500
213	0.0	100	300	400
246	0.0	100	200	300
AVERAGE ..				470

DISCUSSION.

The immunization of a horse for the production of tetanus antitoxin is said to be quite good when its serum has reached the titre 600 I. U. per c.c. It is only in rare cases that horses can be immunized so as to yield sera having a titre higher than 1,400 I. U. per c.c. Predtechensky (1931) claims that by adopting a special method of immunization he could raise the titre of two horses to the high value of 1,500 and 2,000 American units per c.c. Schmidt (1928) records a case in which he obtained as high a titre as 2,940 I. U. per c.c. It will be noticed from the data recorded in Table III that by adopting the procedure already described we have been able to raise the titre of one of our horses above 3,200 I. U. per c.c. and that of another above 2,000 units. It will thus be noted that the titre of 3,200 I. U. per c.c. which has been obtained by us constitutes one of the highest values yet attained in the production of tetanus antitoxin.

CONCLUSION.

Twenty-seven horses were taken for the production of tetanus antitoxin. They were first immunized with plain antigen and then divided into three groups. The first group was again immunized with higher doses of antigen only. The second group, after a period of 10 days' rest, was immunized with alum and antigen. The third group, after a period of 10 days' rest, was treated first with tapioca and antigen and then subdivided into two groups, III-A and III-B, which received respectively CaCl_2 and antigen and MnCl_2 and antigen. Of these four groups—group III-A was found to yield sera of very high titre. In this experiment, MnCl_2 and alum were found to be of little importance for the production of highly potent sera.

ACKNOWLEDGMENTS.

We wish to express our grateful thanks to Dr. B. N. Ghosh, D.Sc., for the suggestion and planning of this work and to the Managing Director, Bengal Immunity, for the facilities placed at our disposal to carry it out.

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VITAMIN-A CONTENTS OF GHEE.

BY

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ONE of us (Grewal, 1934) determined the vitamin-A contents of butter fat prepared under his direct supervision by the Indian method and of butter fat prepared by the modern method from the milk of a known herd. The estimation of vitamin A was made biologically. It was shown that butter fat of butter prepared by the centrifugal method contained more vitamin A than ghee prepared by the Indian method. Preparation of ghee by the Indian method showed a loss of vitamin A.

In this paper the vitamin-A contents of samples of bazaar ghee and of the market butters have been determined. In any such inquiry it is essential to establish the purity of the butter fat. Therefore we determined the following chemical constants of all the samples of ghee :—

1. *The Valenta acetic test.*—This test depends on the intermiscibility of butter fat and strong acetic acid at a low temperature. The animal and vegetable fats (except coco-nut oil) form a clear mixture at a much higher temperature. Strong acetic acid (99 per cent) will give a turbidity with genuine butter fat at from 32°C. to 36°C. Many methods of carrying out the tests have been proposed but the method followed by us was that described in 'Allen's Commercial Organic Analysis', page 384 (Lifemann and Davis, 1910).
2. *The saponification value* is the number of mg. of potassium hydroxide necessary to saponify 1 g. of the fat.
3. *The Helmer value* is the weight of the insoluble fatty acids expressed as a percentage of the fat.
4. *The Reichert-Meissl value* is practically a measure of the butyric and capric acid present. It was determined by us by the method described in 'Allen's Commercial Organic Analysis', page 377 (Lifemann and Davis, *loc. cit.*).

5. *The Polenski' value* indicates the proportion of the glycerides of caprylic, capric, and lauric acids. It was determined by the method referred to above under the R. M. value.
6. *Polenski' limit.*—The Polenski' value rises with the Reichert value. A number of workers have made searching investigations of the variations in the Reichert-Meissl and Polenski' values. Nicholls (1934) suggested two formulas connecting the values; for most practical purposes he proposed the formula $\frac{R-10}{7}$ for the average Polenski' value and $\frac{R-6}{7}$ for the maximum Polenski' value, where R is the Reichert-Meissl value.

The estimation of vitamin A was made by the colorimetric determination of the blue colour of the non-saponifiable fraction of the butter fat with the antimony trichloride reagent. The method used was that of Smith and Hazley (1930) with the modification that 10 g. of the fat were used instead of 2 g. with proportionately larger amounts of the solvents. The chloroform extract was dried over anhydrous sodium sulphate and distilled to a small bulk. The residue was rinsed with dry chloroform into a 5 c.c. or 10 c.c. graduated flask according to the potency of its vitamin-A contents. The natural yellow colour was determined by measuring the yellow colour of the non-saponifiable fraction in chloroform (10 g. in 5 c.c.) with the Lovibond tintometer and the figures in the table indicate the units thus obtained.

Forty-nine samples of butter fats have been examined. Ten of these samples were prepared under direct supervision from the milk of a known herd. The results of these are given in Table I.

Butter fats in Table I give the Carr-Price blue value of 0.58 ± 0.081 . The chemical constants show that they are pure animal fats.

Twelve butters bought from the local market were examined, out of which six were bought in summer and the other six during winter from the same dairies. The results are given in Tables II and III.

In Table II fats give the Carr-Price blue value of 0.36 ± 0.19 . With the exception of butter No. 2, all of them were pure. The usual adulterants, except the coco-nut oil group, give a lower Polenski' value. The high Polenski' value of butter No. 2 suggests its adulteration with coco-nut oil, which is the usual adulterant in the Punjab of that group.

In Table III the fats give the Carr-Price blue value of 0.52 ± 0.12 . Except No. 27 all fats showed a slightly low Polenski' value, other chemical constants being within the normal limits.

The village samples were purchased from villages. The results are given in Table IV.

In Table IV the fats give the Carr-Price blue value of 0.21 ± 0.18 . All the chemical constants, except the Polenski' value which is slightly low, are within normal limits.

Table V gives results of 20 samples of bazaar ghee.

In Table V the fats give the Carr-Price blue value of 0.30 ± 0.17 . The chemical constants show that Nos. 19, 23, 38, 40, and 44 to be very grossly adulterated. The samples gave very little blue colour with the antimony trichloride test. Nos. 13,

20, 33, 35, and 43 show over 30 per cent adulteration with 'Banaspati' ghee, even then two of the samples gave a good degree of blue colour with antimony trichloride. The rest of the samples show a small amount of adulteration.

Table VII gives the analysis of bazaar butters. The summer butters showed a large percentage of water and would have been considered as adulterated according to the British standards.

DISCUSSION.

The composition of butter fat is very variable. Analysis of butter presents great difficulties in the detection of small degrees of adulteration with fats of animal or vegetable nature. In the Punjab this is rendered more difficult by the presence of buffalo butter fat. Buffalo butter fat gives an R. M. value of 32 to 40 which may be as low as 28. As cow butter fat gives an R. M. value of 24 to 32, the buffalo butter fat may be grossly adulterated and may yet yield constants comparable to those of genuine cow ghee. This would add greatly to the difficulty of the working of the 'Pure Food Act'.

Table V shows that very adulterated samples are sold as genuine ghee.

The Polenské values even of the known pure ghee are rather on the low side but the number of the samples examined being small it is difficult to give the significance of this fact. The chief adulterant in the Punjab nowadays is 'Banaspati' ghee which causes greater deviation of the Polenské value than of other chemical constants. It will therefore be profitable to study the relative R. M. and Polenské values of the pure Punjab ghee.

The vitamin-A content is very variable but certainly some of the samples had a fairly large amount. This is a fact of some importance as ghee is probably the chief source of vitamin A in the usual human dietary of the Punjab.

SUMMARY.

1. The Carr-Price blue value of a number of samples of butter and ghee has been determined.

2. The samples of the butter fats prepared at the laboratory gave the Carr-Price blue value of 0.58 ± 0.081 . The market butters in summer had the Carr-Price value of 0.36 ± 0.19 and in winter 0.52 ± 0.12 . All these butters were prepared from cream separated by the modern type of centrifuge. The Carr-Price blue value of ghee collected from villages was 0.23 ± 0.16 and that of samples collected in the city was 0.31 ± 0.17 .

3. The chemical constants of all the fats were determined. It was found that the ghee collected in the city was adulterated, in some cases grossly adulterated although sold as genuine.

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TABLE I.

Butter fat (Lab.).

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	B. refractive index at 40°C.	Saponification value, mg. KOH.	Hehner. value, per cent.	R. M. value, N/10 c.c. KOH.	Polenské value, N/10 c.c. KOH.	Polenské limit calculated according to the formula		Carr-Price blue value in 0.04 g.	Natural yellow colour.	Source.
	Clearing temperature, °C.	Acetic acid absorption, per cent.							$\frac{R-10}{7}$	$\frac{R-6}{7}$			
14	36	60	0.9109	40.2	232	88.1	27.1	2.6	2.44	3.01	0.60	16	Cow's.
15	34	60	0.9112	40.5	229	88.0	26.0	2.6	2.28	2.83	0.60	19	"
45	35	53	0.9110	40.5	232	88.2	27.0	2.6	2.42	3.00	0.60	20	"
46	34	59	0.9111	40.2	232	88.0	26.5	2.6	2.35	2.92	0.64	20	"
16	30	72	0.9118	40.0	229	87.0	32.9	3.0	3.27	3.84	0.48	14	Buffalo's.
17	30	72	0.9128	40.1	230	87.1	34.8	3.3	3.54	4.11	0.52	14	"
25	27	73	0.9118	40.5	224	85.0	34.2	3.0	3.45	4.02	0.74	1.2	"
47	32	72	0.9119	40.0	230	87.0	33.1	3.1	3.30	3.87	0.48	14	"
48	30	70	0.9125	39.5	231	87.1	34.5	3.3	3.50	4.07	0.48	15	"
49	41	60	0.9117	40.0	237	87.7	30.3	3.4	2.90	3.47	0.70	2.4	"

TABLE II.

Butter (summer).

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	B. refractive index at 40°C.	Saponification value, mg. KOH.	Hehner value, per cent.	R. M. value, N/10 c.c. KOH.	Polenské value, N/10 c.c. KOH.	Polenské limit calculated according to the formula		Carr. Price blue value in 0.04 g.	Natural yellow colour.	Source.
	Clearing temperature, °C.	Acetic acid absorption, per cent.							R-10 7	R-6 7			
1	36	60	0.9115	40.0	235	85.6	32.4	2.0	3.20	3.78	0.50	0.7	Bazaar.
2	44	54	0.9115	41.0	227	87.8	31.3	4.3	3.04	3.61	0.08	0.2	"
3	35	54	0.9116	40.0	234	86.8	28.8	2.3	2.68	3.25	0.60	2.0	"
4	32	60	0.9126	40.0	239	86.6	31.6	3.0	3.08	3.65	0.44	0.7	"
5	35	62	0.9112	40.5	230	86.3	28.9	3.3	2.70	3.27	0.12	0.7	"
18	32	58	0.9118	40.0	227	87.0	32.4	3.1	3.20	3.77	0.40	2.4	"

TABLE III.

Butter (winter).

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	B. refractive index at 40°C.	Saponification value, mg. KOH.	Hegner value, per cent.	R. M. value, N/10 c.c. KOH.	Polenské value, N/10 c.c. KOH.	Polenské limit calculated according to the formula		Carr-Price blue value in 0.04 g.	Natural yellow colour.	Source.
	Clearing temperature, °C.	Acetic acid absorption, per cent.							$\frac{R-10}{7}$	$\frac{R-6}{7}$			
26	34	60	0.9125	40.0	231	88.8	33.1	2.6	3.30	3.87	0.41	1.0	Bazaar.
27	36	57	0.9118	40.0	225	86.0	28.8	2.8	2.68	3.25	0.37	20.0	"
28	38	65	0.9123	40.5	240	86.8	31.7	2.1	3.10	3.67	0.49	2.5	"
29	34	73	0.9124	39.5	227	85.0	34.0	2.9	3.42	4.00	0.57	2.6	"
30	38	70	0.9111	40.0	238	87.0	31.0	2.6	3.00	3.57	0.56	2.6	"
31	41	70	0.9117	40.0	237	88.7	30.3	2.4	2.90	3.47	0.70	..	"

TABLE IV.

Ghee (village).

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	B. refractive index at 40°C.	Saponification value, mg. KOH.	Hehner value, per cent.	R. M. value, N/10 c.c. KOH.	Polenské value, N/10 c.c. KOH.	Polenské limit calculated according to the formula		Carr-Price blue value in 0.04 g.	Natural yellow colour.	Source.
	Clearing temperature, °C.	Acetic acid absorption, per cent.							R-10 7	R-6 7			
6	40	62	0.9126	40.5	223	87.6	26.9	2.3	2.41	2.98	0.24	0.7	Village.
7	39	60	0.9115	42.0	225	88.6	25.0	2.0	2.14	2.71	0.08	0.3	"
8	45	60	0.9114	41.5	229	89.4	28.8	2.8	2.68	3.25	0.14	0.6	"
9	0.9116	39.0	228	85.3	27.7	2.4	2.52	3.10	0.14	9.0	"
21	47	43	0.9118	41.0	230	86.9	27.4	2.1	2.48	3.05	0.50	0.9	"
24	35	50	0.9113	39.5	224	80.7	29.2	2.2	2.74	3.31	0.36	..	"
39	30	100	0.9121	40.0	230	84.2	34.1	1.6	3.44	4.01	0.02	..	"

TABLE V.
Ghee (bazaar).

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	B. refractive index at 40°C.	Saponification value, mg. KOH.	Hehner value, per cent.	R. M. value, N/10 c.c. KOH.	Polonské value, N/10 c.c. KOH.	Polonské limit calculated according to the formula		Carr-Price blue value in 0.04 g.	Natural yellow colour.	Source.
	Clearing temperature, °C.	Acetic acid absorption, per cent.							$\frac{R-10}{7}$	$\frac{R-6}{7}$			
10	35	52	0.9123	40.5	235	88.8	33.1	2.0	3.30	3.87	0.36	6.0	Bazaar.
11	55	40	0.9108	41.0	236	90.0	28.1	1.1	2.58	3.15	0.38	6.0	"
12	52	44	0.9112	40.5	226	89.0	28.3	1.3	2.61	3.18	0.08	2.0	"
13	52	44	0.9117	41.0	217	91.0	26.7	1.1	2.38	2.95	0.06	0.2	"
19	77	33	0.9085	49.0	206	92.2	7.8	0.5	Nil	0.25	Nil	Nil	"
20	52	40	0.9100	40.5	216	87.9	24.8	1.1	2.11	2.68	0.45	1.1	"
22	39	50	0.9115	39.5	232	87.5	31.0	1.7	3.00	2.57	0.34	1.2	"
23	55	33	0.9100	40.5	216	88.1	20.4	1.1	1.48	2.05	0.24	11.1	"
32	40.5	72	0.9115	41.5	219.2	87.8	27.9	1.45	2.56	3.13	0.30	..	"
33	46.5	60	0.9129	40.1	224.7	87.1	26.0	1.55	2.30	2.85	0.50	..	"
34	36.0	80	0.9116	40.0	226.0	87.5	28.6	1.65	2.65	3.22	0.40	..	"
35	42.5	67	0.9098	43.3	219.0	87.5	24.4	0.85	2.05	2.63	0.42	..	"
36	28.0	100	0.9123	41.0	226.1	86.8	32.0	1.80	3.14	3.71	0.40	..	"
37	30.5	96	0.9118	41.2	223.2	86.2	29.6	2.80	2.80	3.37	0.65	..	"
38	81.0	40	0.9058	48.0	197.7	92.7	7.4	0.30	..	0.20	Nil	..	"
40	59.0	53	0.9090	45.5	213.8	88.0	18.9	1.05	1.27	1.84	0.05	..	"
41	28.7	100	0.9122	40.0	233.5	86.5	37.6	1.83	3.94	4.51	0.30	..	"
42	36.5	89	0.9110	40.1	233.0	85.6	31.7	1.35	3.10	3.67	0.10	..	"
43	38.5	78	0.9114	41.1	222.8	88.6	26.6	1.95	2.37	2.94	0.35	..	"
44	62.0	53	0.9084	46.5	211.4	91.8	16.8	0.70	0.97	1.54	0.04	..	"

TABLE VI.

Vegetable ghee.

Number of sample.	VALENTA TEST.		Sp. gr. at 40°C.	R. refractive index at 40°C.	Saponification value, mg. KOH.	Hehner value, per cent.	R. M. value, N/10 c.c. KOH.	Polenské value, N/10 c.c. KOH.
	Clearing temperature, °C.	Acetic acid absorption, per cent.						
51	91	40	0.9041	50.5	192.4	92.3	0.33	0.05
52	50	42	0.9043	50.0	187.7	95.3	0.44	0.10
53	50	40	0.9042	50.2	187.7	93.5	0.16	0.05
54	50	42	0.9045	40.1	190.0	93.4	0.27	0.05

TABLE VII.

Analysis of bazaar butters of which the analysis of fats is given in Tables II and III.

Number of sample.	Mixture, per cent.	Card, per cent.	Salt, per cent.	Colour.
1	29.7	1.81	Nil	Nil
2	17.7	1.38	"	"
3	26.7	1.49	"	"
4	22.6	1.40	"	"
5	17.0	1.19	1.16	"
18	19.9	1.20	Nil	"
25	17.0	1.10	"	"
26	19.0	1.20	"	"
27	15.6	1.50	"	"
28	15.2	2.00	"	"
29	16.4	1.40	0.1	"
30	12.5	1.00	1.0	"

STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part I.

THE CALCIUM AND PHOSPHORUS CONTENT OF THE SOFT TISSUES OF NORMAL RATS.

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INTRODUCTION.

THE major portion of the body-weight of an animal consists of bones, and these contain about 99 per cent of the calcium found in the body. The remainder is distributed between the soft tissues and the blood. The calcium content of the latter has been extensively investigated and a fair amount of knowledge gained about the normal amount of blood calcium, the state in which it exists, and the various conditions which influence both. The soft tissues, however, have received comparatively little attention, perhaps, because the amount of calcium in the tissues has been found to be small and extremely variable. So far as the blood is concerned the amount of calcium circulating in it is, for a particular species of animals, constant in health and unaffected in many diseases. It is appreciably altered, however, in low calcium rickets, osteomalacia, parathyroid tetany, hyperparathyroidism, chronic glomerular nephritis, and nephrosis (Schmidt and Greenberg, 1935). It is possible that the calcium content of the tissues other than blood may likewise be altered by diseases characterized by a disturbance in the calcium metabolism. That it is so altered, at least, in rickets has been shown by Hess, Gross, Weinstock and Berliner (1932) for the brains of rachitic rats and by Haury (1930) for the striated muscle also of rachitic rats. Further, Reed, Dillman, Thacker and Klein (1933) have shown that on administration of toxic doses of irradiated ergosterol the calcium content of the soft tissues of dogs was significantly increased, while the change observed in their phosphorus content was rather small.

The data about the level of calcium and phosphorus in the tissues of normal animals are scanty. Heubner and Rona (1923) as well as the other workers quoted

above have reported gross variations in the amounts of these elements in the soft tissues of several species of normal animals. Denis and Corley (1925) suggest, however, that the inconstancy might be partly due to the difficulty of obtaining a true sample of tissue free from interstitial fat, blood, and connective tissue. Burns (1933) showed that in the case of muscle calcium, fat, nerve or connective tissue would need be present to the extent of 50 to 60 per cent of the weight of the muscle to appreciably influence the variation in its calcium content; hence she concluded that it was improbable that the major variation observed in muscle calcium could be at all ascribed to contamination with other soft tissues. Burns further finds that the muscle calcium of rats is not significantly altered in rickets and parathyroid tetany.

In view of these facts it was felt that some more information about the calcium and phosphorus in the soft tissues of normal animals was necessary for the interpretation of the observed changes due to diseases such as rickets, osteomalacia, tetany, etc. Moreover, besides the amounts of these elements the state in which they exist in the tissues and their relation to the other tissue constituents are other important aspects about which some definite information is desirable. Hence it was decided to make a start with the estimation of calcium and phosphorus in the soft tissues of carefully selected normal rats, the other aspects of this large question to be taken up later in the course of these investigations.

EXPERIMENTAL.

Since the calcium content of the body of an animal could be markedly influenced by its dietary history (Sherman *et al.*, 1936) it was thought that a comparison of the calcium and phosphorus content of the soft tissues of rats of the same age fed for a sufficient length of time on the same diet would show less variation than has been observed by the previous workers; the experiments reported below seem to have only partly justified this assumption.

The strain of albino rats used in these experiments was obtained from the Pasteur Institute, Coonoor, South India, and bred locally for over two years. Young rats of 28 to 30 days from several litters were kept on diet I (Ca 0.390 per cent, P 0.315 per cent, and Ca : P 1.23 per cent) for 17 weeks. At the end of this period some of them were subjected to a study of Ca and P balance.

Ca and P balance.—The rats were kept in metabolic cages which were fixed over large glass funnels fitting well into the base of the cages. The stems of the funnels were packed lightly with glass wool and the lower ends of the stems opened in glass bottles for the collection of urine. A few c.c. of hydrochloric acid were added to each bottle to prevent putrefaction. The faeces were collected every day from the cages and from the funnels. Food and water were given *ad lib.* The food was supplied in the form of a coarse powder and the containers were such that the food could not be spilt outside them. Every day a weighed quantity of food was kept in the cages; another weighed sample was dried and the amounts of Ca and P estimated. The residue from the previous day was similarly treated and analysed. Thus, the amount of dry food consumed as well as the actual daily consumption of Ca and P were found out. The urine and the faeces collected over four days were pooled before analysis. The urines were made to a known volume and aliquot portions ashed. Faeces were dried, powdered, and ashed. The calcium

was estimated by precipitation as oxalate and its titration with standard KMnO_4 . Phosphorus was estimated by the Bell and Doisy (1920) modification of Brigg's method.

Two more batches of six rats each had been kept for 24 weeks on two different diets, designated here as diets II and III, for an entirely different purpose. It seemed reasonable, however, to use them for this investigation since the Ca and P contents of these diets happened to be very much similar to that of diet I. The composition of these diets is given in Table I:—

TABLE I.
The composition of the diets.

Article.	DIET I.	Article.	DIET II.	DIET III.
	Quantity in g.		Quantity in g.	Quantity in g.
Wheat flour ..	420	Wheat flour	170	170
Gram flour ..	140	Polished rice	170	170
Ankoria babyfood ..	35	Jowar (<i>Sorghum vulgare</i>)	85	..
Sesame oil ..	35	Bajri (<i>Pennisetum typhoi-</i>	..	85
Meat paste ..	14	deum).
Calcium carb. ..	3.5	Tur dahl (<i>Cajanus indicus</i>)	28	28
Sodium chloride ..	3.5	Soya beans	28
		Rice bran	28
		Ground-nut cake	14
		Meat and fish ..	85	85
		Eggs	28	..
		Skim milk (dried) ..	21	28
		Sesame oil	28	35
		Butter	21	14
		Sugar	57	57
		Vegetables	227	227
Fats	7.5		12.2	11.5
Proteins	15.5		13.6	15.3
Carbohydrates ..	77.0		74.2	73.2
Calcium	0.390		0.380	0.336
Phosphorus ..	0.315		0.345	0.315
			Per cent.	Per cent.
Ca : P	1.23		1.10	1.06

The various articles of the diet were mixed with an adequate amount of water and cooked. The cooked food was dried in a current of hot air at 45°C . and powdered. This amount was sufficient to feed six rats for seven days. Milk was supplied fresh each day. The rats on diet I received 10 c.c. of milk per rat per day, those on diet II 8 c.c., and rats on diet III 4 c.c. per rat per day. The figures for calcium and phosphorus mentioned in the table are inclusive of milk. Four rats from diet group I and two from diet groups II and III each were subjected to Ca and P balance study. The results are given in Table II:—

TABLE II.
Ca and P balance.

Rat number and sex.	Weight. g.	Diet.	Ca : P.	CALCIUM IN MG.			PHOSPHORUS IN MG.			
				Intake, mg.	Excretion in		Intake, mg.	Excretion in		Balance, mg.
					Urine, mg.	Faeces, mg.		Urine, mg.	Faeces, mg.	
350 ♀	119	I	1.22	38.50	2.23	8.03	30.64	3.53	10.35	+ 16.76
355 ♂	153	"	1.22	36.60	2.52	12.68	29.10	3.63	9.16	+ 16.31
359 ♂	129	"	1.22	38.46	3.29	6.48	30.62	4.10	9.75	+ 16.77
361 ♂	158	"	1.22	37.41	4.26	8.14	29.59	4.16	8.36	+ 17.07
328 ♂	205	II	1.09	37.35	1.62	13.89	33.99	6.95	10.55	+ 16.49
329 ♂	215	"	1.10	36.10	1.16	12.42	32.77	6.16	10.74	+ 15.87
338 ♂	235	III	1.06	33.57	1.44	25.90	31.52	5.10	18.10	+ 8.32
241 ♀	155	"	1.09	32.63	1.35	24.80	29.92	3.02	19.89	+ 7.01

Analyses of the tissues for Ca and P.—It was concluded from the balance experiments that the calcium and phosphorus requirements of these rats and also of those others which were kept under identical conditions but not submitted to balance tests were being met from the food, and hence the conditions were deemed favourable for a comparison of the Ca and P content of the tissues, as the variations in the amounts of these elements were less likely to occur than might be expected in a condition of negative Ca and P balance.

With a view to minimize errors due to a possible contamination with blood the rats were bled in the earlier experiments by the introduction of a cannula in the abdominal aorta under ether anaesthesia before being killed for the removal of the soft tissues. But the amount of blood obtained was never more than 3 c.c. Further as it was felt that the draining of blood by saline, even if possible, would remove not only blood but some of the calcium and phosphorus from the tissues as well, the procedure was not attempted and bleeding by cannula discontinued. The rats were killed by chloroform, the soft tissues—muscle (thigh), liver, spleen, kidney, testes, heart, lungs, and brain—were removed as quickly as possible, freed from extraneous blood by gently pressing between filter-papers and put into previously weighed weighing bottles. These were weighed again and dried together with the contents in a water oven to constant weight. For analysis each tissue was transferred as completely as possible to a weighed platinum dish; a second weighing gave the actual weight of the tissue used. It was then ashed according to Stolte's method as described by van Slyke and Peters (1932). The ash was dissolved in dilute hydrochloric acid, the solution made to a known volume and aliquot portions used for Ca and P estimations. The calcium was estimated according to the method of Wang (1935) and phosphorus by the modified Brigg's method.

Six rats were analysed from each diet group. The results calculated on the moisture-free tissues are given in Tables III and IV. The moisture content for all tissues examined varied between 70 and 80 per cent. These tables also include analyses of the tissues of six rats dead through starvation. Reference to these experiments will be made later.

For the sake of brevity the values for all the tissues of all the rats examined have not been given; only the maximum, minimum, and mean values being given together with the standard deviation.

3

DISCUSSION.

Calcium in the soft tissues.—A comparison between the tissue Ca of the normal rats of all the three diet groups shows that the mean values for calcium in most cases vary from group to group. Among the rats of the same group the variation is smallest as indicated by the maximum and minimum figures for each tissue. A somewhat close approximation is shown between the groups B and C (Table III). This might be due to many factors among which might be included the almost identical ratio of Ca : P in diets II and III and a similarity in age. The rats in groups B and C had an average age of 200 days, while those in groups A and D were 150 days old. But even between groups B and C variations are to be found in spite of the similarity of age and Ca : P ratio, for not only the mean values but also the maximum and minimum values for Ca are different for spleen, testes, and hearts

of rats belonging to these two groups. It is evident that the diet has influenced the Ca content of the tissues. The influence might possibly be due to the vitamin-D content of the diets used, a possibility which requires further testing. A reference to Table II will show that, although the Ca : P ratios were not very much different for the three diets, Ca and P retentions were not the same in each group. The figures for tissue Ca and P, however, do not point to any relation between Ca and P retention and the amount of these elements found in the soft tissues. The values for muscle calcium reported in this paper are lower than those found by Haury (*loc. cit.*). The somewhat higher figures for calcium in the muscles of both the normal and rachitic rats obtained by Haury can be explained by the fact that this investigator extracted the muscle with fat solvents before analysis, the amount of calcium then being expressed on fat-free muscle. A similar procedure has been followed by Hess *et al.* (*loc. cit.*) who reported a very high figure for brain calcium.

Certain facts, however, emerge clearly from these results (Table III). The lungs seem to contain the largest and the liver the least amount of calcium found in the soft tissues so far examined irrespective of the diet. The brain is next to the lungs in its Ca content. In case of the liver the mean value of calcium was found to be independent of the diet and was not altered even in extreme starvation.

Phosphorus in the soft tissues.—The amount of total phosphorus (Table IV) in the tissues was found to be very much larger than that of calcium as is to be expected from the fact that phosphorus is more intimately linked with the structure of the protoplasm than calcium. In this case also large variations were observed, and the data are less liable to even as clear an interpretation as in the case of calcium. It seems, however, that the brain, spleen, testes, and lungs contain more total phosphorus than the other tissues examined. In starvation there appears to be an all-round reduction in total phosphorus in the tissues with the probable exception of the brain.

The Ca and P content of the soft tissues of rats in extreme starvation.—Six rats used for this experiment had been kept on diet I for 17 weeks under conditions identical with those for the rats of group A. When they were 150 days of age food was withheld from them but water was allowed. The death took place within six to eight days. Their weight loss and nitrogen, calcium and phosphorus excretion were studied. But the figures have not been included here because no relation could be established between the excretion of N, Ca, and P nor could any inference be drawn about the excretion of Ca and P in relation to their amounts in the tissues. After death the soft tissues were removed and analysed as in the case of other rats. The results are included in Tables III and IV under group D.

These experiments were designed to investigate whether by starvation the tissue Ca could be brought to a level characteristic of any particular tissue and representing a functional level. The conception of an 'élément variable' and an 'élément constant' in relation to the fat content of tissues has gained wide acceptance due to the work of Terroine (1927) and others. The starvation experiments were an attempt to find out analogous relations for the calcium in the tissues. The experiments, however, have failed to yield the desired information.

TABLE III.

The calcium content of the soft tissues of rats.

Mg. Ca per 100 g. dried tissue.

	Muscle.	Brain.	Spleen.	Testes.	Liver.	Heart.	Lungs.	Kidney.
Group A, Diet I.	Maximum ..	32.3	65.01	41.88	38.7	61.7	145.6	62.5
	Minimum ..	65.3	36.92	38.58	21.1	51.5	97.1	47.6
	Average ..	79.59	55.74	40.09	28.83	53.01	121.3	53.07
	σ ..	4.01	4.09	..	3.098	1.57	7.92	2.12
Group B, Diet II.	Maximum ..	36.4	46.4	64.0	35.4	43.2	84.9	34.8
	Minimum ..	53.9	32.6	43.4	27.4	35.2	59.8	27.5
	Average ..	57.65	38.72	52.80	31.87	38.50	72.25	30.05
	σ ..	1.90	2.39	..	1.17	1.45	4.14	1.07
Group C, Diet III.	Maximum ..	73.8	61.9	40.3	35.6	56.6	119.2	38.8
	Minimum ..	45.4	42.2	23.3	22.6	41.2	56.3	29.1
	Average ..	53.53	52.70	32.47	28.30	49.15	80.53	32.70
	σ ..	3.77	3.75	..	2.70	2.74	12.56	1.86
Group D, Starved rats.	Maximum ..	115.3	144.0	50.1	43.6	98.2	123.6	45.9
	Minimum ..	25.6	99.6	22.5	14.6	16.2	89.5	22.8
	Average ..	56.25	123.8	41.84	28.03	49.38	105.2	35.03
	σ ..	15.03	10.02	4.02	3.84

TABLE IV.
The phosphorus content of the soft tissues of rats.
 Mg. P per 100 g. dried tissue.

	Muscle.	Brain.	Spleen.	Testes.	Liver.	Heart.	Lung.	Kidney.
GROUP A, <i>Diet I.</i>	Maximum ..	1,169	1,557	1,741	1,576	1,165	1,118	1,250
	Minimum ..	818	1,000	1,346	1,315	834	714	1,123
	Average ..	956	1,344	1,480	1,476	965	881	1,211
	σ ..	47.84	82.60	63.78	..	59.36	69.98	18.97
GROUP B, <i>Diet II.</i>	Maximum ..	928	1,544	1,547	1,469	1,014	1,116	1,144
	Minimum ..	742	1,199	1,156	1,197	792	927	891
	Average ..	809	1,362	1,291	1,303	939	1,001	1,021
	σ ..	41.33	55.38	64.92	..	34.35	30.45	34.10
GROUP C, <i>Diet III.</i>	Maximum ..	885	1,662	1,608	1,401	929	1,083	1,015
	Minimum ..	755	1,581	1,214	1,272	688	813	830
	Average ..	831	1,626	1,406	1,335	863	953	915
	σ ..	27.45	16.25	56.6	..	32.86	32.66	36.3
GROUP D, <i>Starved rats.</i>	Maximum ..	824	1,655	1,524	1,936	1,057	891	991
	Minimum ..	656	1,158	1,015	889	864	524	894
	Average ..	751	1,325	1,288	1,302	950	749	958
	σ ..	31.99	69.18	85.9	..	24.36	69.2	15.4

Since the diet and the conditions of living of the rats in group D were identical with those of rats in group A prior to the starvation of the former, the figures for Ca and P in the tissues of the starved rats should be compared with those of rats of group A. The figures in Table III reveal a large increase (120 per cent) in calcium in the spleen and a much smaller one in the muscles of the starved rats. The calcium in the kidney appears to have decreased owing to starvation. The changes in the phosphorus content (Table IV) in starvation were small in magnitude and were confined to the muscle, lungs, and kidney.

During starvation the tissue proteins are broken down and consequently the tissues lose in weight. In the present experiments the spleens suffered a loss of 65 per cent on the average and the livers 53 per cent and yet the Ca content of the spleens was more than doubled in extreme starvation, while that of the liver was hardly affected. It has not been possible to offer an adequate explanation of the observed accumulation of calcium in the spleen of the starved rats.

Further work on the effect of rickets and allied conditions and of dosage with irradiated ergosterol on the Ca and P content of the tissues and on the distribution of phosphorus in its various combined forms is in progress and will form the subject of later communications.

SUMMARY.

The soft tissues of normal rats kept on three different diets from 17 to 24 weeks have been examined for their calcium and phosphorus content. The soft tissues of rats dying of extreme starvation have also been similarly analysed. From the results which have been statistically treated the following conclusions can be drawn :—

- (1) Large variations in the amount of calcium and phosphorus in the soft tissues of normal rats have been observed. The diet seemed to influence the mean values of Ca and P, different mean values being obtained for each of the diet groups. The difference between the maximum and minimum values was found to be least among the tissues of rats belonging to the same diet group.
- (2) The Ca : P ratio of the diets showed no relation to the mean levels of Ca and P in the tissues.
- (3) Irrespective of the diet, the lungs contained the highest amount of calcium of all the tissues examined, and the liver the least. The amount of calcium in the brains was found to be next largest to that in the lungs. The amount of phosphorus was found to be greater in the brain, spleen, testes, and lungs than in the other tissues examined.
- (4) In extreme starvation calcium in the spleens of starved rats increased by 120 per cent (average) over that found in the spleens of the normal animals.
- (5) The mean value for calcium in the liver of the normal rats was unaltered by change of diet or in starvation.

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THE TREATMENT OF STOMATITIS CAUSED BY DIET DEFICIENCY.

BY

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WE have previously described (Aykroyd and Krishnan, 1936) a type of stomatitis occurring in South India, which is very common in malnourished children. It has been pointed out that this condition appears to be essentially similar to the stomatitis observed in pellagra, though it is, in our experience, not accompanied by other signs characteristic of pellagra such as diarrhoea and dermatitis. We described its rapid cure by dried yeast, unheated and autoclaved, and skimmed milk, and pointed out that the most likely cause of the condition was deficiency of one or more of the factors present in vitamin-B₂ complex. In a later paper (Aykroyd and Krishnan, 1937) an investigation of the incidence of stomatitis in a series of residential institutions for children was reported. This investigation showed that there is a clear-cut relationship between the disease and the quality of the diet consumed; it is found only when the diet is composed almost exclusively of milled rice and contains almost negligible quantities of vegetables and other foods. Further experience has fully confirmed the truth of this observation. If stomatitis is present in a boarding school, the diet is invariably found to be of the type described, and it is a simple matter to eradicate it by suitable dietary modifications.

The rapid response of the disease to treatment makes it possible to study fairly easily the effect of different foods. In the present paper we report the results of giving eggs, soya bean, and yeast autoclaved in an alkaline medium to children suffering from stomatitis.

THE TREATMENT OF STOMATITIS.

The following is a general description of the cases treated:—

White patches were present at the corners of the mouth; closer inspection usually revealing an ulcerated fissure. The mucous membrane of the tongue was red and eroded, this being particularly marked at the sides and tip. Fissures were sometimes present on the surface of the anterior part of the tongue. The patient complained that the mouth was 'sore', and eating was usually painful.

The earliest effect of successful treatment is a disappearance of the sensation of pain. This may occur in a few days. Subsequently the patches and erosions at the labial angles disappear and the tongue assumes a normal appearance. Fissures on the tongue may persist after the rawness has disappeared.

The cases given treatment were all boys of ages ranging from 8 to 16. Nearly all were living in boarding schools, and during the period the supplement was given they continued to consume the diets which had led to the development of stomatitis. These diets were of the defective type previously described (Aykroyd and Krishnan, 1937); i.e., they were largely composed of milled rice and contained little or no milk, and inadequate amounts of vegetables.

Eggs.—Seven boys in all were treated, three in one institution and four in another. In three cases, one egg daily for 32 days produced definite improvement, but not complete disappearance of all signs. Two eggs were then given daily for 20 days, and complete cure resulted. The four boys given three eggs daily were cured in three weeks.

Other pupils in the institutions on the same diet without the addition of eggs showed no improvement during the same period.

Soya bean.—Five cases in a boarding school received 1·5 oz. daily of soya bean for 32 days and 3 oz. daily for 32 days. The beans were given whole, being cooked until soft. No improvement was observed in any of the boys, and one case became worse. In the same period three boys in the same school were cured by eggs.

Alkaline autoclaved yeast.—The yeast given was autoclaved for five hours at 130°C. at pH 9·2 and subsequently neutralized; this procedure destroys vitamin B₁ and the flavin component of the vitamin-B₂ complex. The absence of flavin was confirmed by chemical test. Eight boys in all were treated, the dose being 0·5 oz. per day. Of four cases in a boys' hostel, three were completely cured after receiving 26 oz., while the remaining case showed definite improvement. The effect of treatment on four day-school boys in Coonoor and neighbourhood was also observed. These boys belonged to the poorest classes and their diet was largely milled rice; it could justifiably be assumed that no change in the diet consumed at home was likely to occur during the period of treatment. The improvement when the yeast (0·5 oz. daily) was given was immediate and unquestionable. Within a few days soreness of the mouth had disappeared and the boys expressed their delight at the improvement. After 10 oz. to 14 oz. of yeast had been taken signs of stomatitis had completely disappeared in three cases, and were only slightly visible in the fourth.

DISCUSSION.

The following summarizes the existing knowledge about the distribution of the 'anti-stomatitis' vitamin in foodstuffs, based on clinical observation :—

Milled rice	Absent.* †
Millet	Present.†
Milk, including skimmed milk	Abundantly present.*
Yeast (unheated)	" " * §
Yeast (autoclaved)	" " * §
Yeast (autoclaved in alkaline medium)	" " †
Eggs	" " †
Soya bean	Poor or lacking in the factor.‡
Liver	Abundantly present.§

* Aykroyd and Krishnan (1936).

‡ Present investigation.

† *Idem* (1937).

§ Landor and Pallister (1935).

It will be observed that the distribution of the 'anti-stomatitis' factor, in so far as it is at present known, appears to correspond to that of pellagra-preventive (P-P) factor. The observation that alkaline autoclaved yeast cures stomatitis is of importance. Yeast so treated lacks flavin, so that flavin can be eliminated as the responsible factor. Similarly it has been observed that flavin has no curative effect on pellagra, nor does it cure black-tongue in dogs (Dan, 1936; Birch, György and Harris, 1935).

It seems unlikely that stomatitis is due to deficiency of vitamin B₆. This vitamin is apparently present in abundance in whole cereals (Birch *et al.*, *loc. cit.*). Copping (1936) found that there was loss of vitamin B₆ from cereals on milling, but reported that milled cereals, including milled rice, contained fair quantities of the vitamin. According to figures given by Birch and his co-workers, skimmed milk, which is effective against stomatitis, is not a rich source of vitamin B₆.

The 'anti-stomatitis' vitamin is probably identical with the factor in the vitamin-B₂ complex which is effective in treating human pellagra (Fouts, Lepkovsky, Helmer and Dukes, 1936), and which cures black-tongue (Koehn and Elvehjem, 1937). It seems more than probable that the factors which have a curative effect on pellagra, stomatitis and black-tongue are one and the same. If this is so, we are faced with the problem why the consumer of milled rice, in contradistinction to the maize-eater, develops only one symptom of pellagra, and escapes the rest. Stomatitis when severe may be unpleasant and its victims are usually in a very poor 'state of nutrition'. Nevertheless it cannot, as *usually* observed, be described a serious condition, while pellagra is a formidable disease with a heavy mortality.

SUMMARY.

1. Stomatitis occurring in children living on a diet largely composed of milled rice can be cured by eggs and yeast autoclaved in an alkaline medium. Soya bean has no curative effect.

2. It seems probable that the factor which cures stomatitis is identical with the P-P factor.

ACKNOWLEDGMENTS.

We are indebted to the superintendents and headmasters of various schools who co-operated in these investigations.

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SKIMMED MILK AND THE GROWTH OF SCHOOL CHILDREN.

BY

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WITH A STATISTICAL NOTE

BY

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IN an earlier series of experiments (Aykroyd and Krishnan, 1937), the effect of adding liquid skimmed milk reconstituted from powder to the diet of children in residential hostels was investigated. The result was an acceleration in growth and a marked improvement in general condition. The present paper describes investigations similar in principle but carried out with day-school children of the poorer classes. The children in the residential hostels were consuming diets of known composition, apparently sufficient to cover calorie requirements, whereas little information was available about the diets of the children included in the present investigation beyond the fact that they were probably deficient in quality and quantity. The investigation of the effect of giving milk to children not living in institutions was of obvious importance. A further point of novelty was that, in one of the experiments recorded here, liquid separated milk of local origin was used instead of milk reconstituted from imported powder. Owing to expense and other considerations, the number of children included in these experiments was limited.

EXPERIMENT A.

This investigation was carried out in a poor class area in one of the suburbs of Madras City. Forty boys were selected and were divided at random into two groups of 20 each. The boys appeared to be typical of the class of children living

in the locality, their 'state of nutrition', as evidenced by general appearance and the presence of stomatitis, being extremely poor. Their ages lay between six and twelve. In the first group each boy received daily 8 oz. of liquid skimmed (separated) milk supplied by the Co-operative Milk Supply Union in Madras, while the boys in the other group did not receive any supplement to their diet. The experiment was carried on continuously for three months during the hot season. One boy in the group not receiving milk failed to appear when the group was being weighed at the end of the experiment.

The boys were weighed and measured at the beginning and end of the three months' period, an Avery's lever balance being used. Increases in height and weight were as follows:—

GROUP I. Skimmed (separated) milk group—

Average increase in weight = 1·91 lb.

Average increase in height = 0·66 inch.

GROUP II. (Not receiving separated milk)—

Average increase in weight = 0·84 lb.

Average increase in height = 0·37 inch.

The difference in the weight and height increments in the two groups is significant (vide *Appendix A*).

EXPERIMENT B.

This experiment was organized to provide a demonstration of school-feeding methods and of the value of a milk supplement, for a group of Health Officers from various provinces undergoing a training course in nutrition in Coonoor.

Boys and girls from a day school in Coonoor situated close to the Laboratories were selected for the experiment. They belonged to the families of poor labourers or domestic servants, and it is probable that the diet they consumed at home consisted largely of milled rice. Forty-eight boys and thirty-six girls were collected for the experiment. The boys were divided at random into two groups of 24 each and the girls into two groups of 18 each. Their ages ranged from six to twelve. One group of boys and one group of girls were given 8 oz. of skimmed milk reconstituted from powder daily, while the second group of boys and girls was given 1 oz. of ordinary biscuits composed chiefly of refined wheat flour, yielding about the same number of calories as the reconstituted skimmed milk. The experiment was carried on for 10 weeks. The children were weighed and measured at the beginning and end of the period. Increases in weight and height were as follows:—

Boys: Skimmed milk group—

Average increase in weight = 1·53 lb.

Average increase in height = 0·61 inch.

Biscuit group—

Average increase in weight = 0·09 lb.

Average increase in height = 0·33 inch.

These differences are statistically significant (vide *Appendix B*).

Girls: Skimmed milk group—

Average increase in weight = 1.31 lb.

Average increase in height = 0.67 inch.

Biscuit group—

Average increase in weight = 0.90 lb.

Average increase in height = 0.37 inch.

Two girls in the group receiving milk left the school during the experiment and hence the number in this group dropped to 16. The difference in the height increments was significant statistically, the difference as regards weight increases being non-significant (girls).

In all the experiments, the children receiving the skimmed milk showed an evident improvement in general condition.

It costs about Re. 0-12-0 per month to supply a child daily with 8 oz. of liquid milk reconstituted from skimmed milk powder. The 8 oz. of fresh skimmed milk given in Experiment A cost about the same, Re. 0-11-3, per child per month. The present experiments confirm the earlier ones carried out in boarding schools and show that skimmed milk will benefit day-school children who may be consuming an insufficient and unsatisfactory diet in their homes, and it is felt that this method of school feeding may be strongly recommended to educational authorities. Undiluted butter-milk has the same nutritive value as the fresh skimmed milk given in Experiment A.

SUMMARY.

The addition of fresh liquid skimmed milk and milk reconstituted from powder to the diet of day-school children produced an enhancement of growth and an improvement in general condition.

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A STATISTICAL NOTE ON THE EXPERIMENTS WITH SKIMMED MILK.

BY

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APPENDIX A.

Experiment A.

THE average heights and weights in the two groups of boys at the beginning of the experiment were not identical, but the statistical constants of the attributes do not differ significantly as would appear from Table I. In spite of the subtle disabilities associated with the verdict 'not significant', there seems to be a likelihood of both the groups being sampled at random from the same infinite population.

TABLE I.

*Analysis of the statistical constants obtained from the grouping of the
initial heights and weights.*

	WEIGHT IN POUNDS.		HEIGHT IN INCHES.	
	S group.	N-S group.	S group.	N-S group.
Number in the sample ..	20	19	20	19
Mean of the sample ..	45.8750	43.4737	48.2375	47.4211
Standard error of the mean ..	2.0832	2.0685	0.8397	0.9430
Difference between the two means	2.4013		0.8164	
Standard error of difference ..	2.9484		1.2623	
<u>Difference</u> S. E. of difference	0.8144		0.6462	
Significance	Not significant.		Not significant.	

One group consisting of 20 boys (skimmed milk group=S) were put on skimmed milk, and another group, comprising 19 boys (non-skimmed milk group=N-S) served as control. The alterations in height and weight observed at the end of three months have been analysed, and are shown in Table II. It is quite apparent that the S group gained more height and weight than the N-S group, and the deviation observed in the case of each of the attributes is significant statistically. Table III shows the variations of the true mean of the height and weight increments in the N-S group, and the extent by which the sample mean of the S group exceeds the maximum range of the variation of the means in the former group.

An attempt was made to find out if there was any association between the increment of weight and increment of height in each of the groups separately. The biserial r for the S group was -0.0371 ± 0.2236 , and that for the other group -0.0267 ± 0.2294 . In other words, the increment of height in each of the groups was not responsible for the gain in weight observed.

Consequently, on statistical grounds alone, it may be safely presumed that the boys fed on skimmed milk put on more weight and height than the boys in the control series.

TABLE II.

Analysis of the statistical constants obtained from the increments of height and weight in the two groups at the conclusion of the experiment.

	INCREMENT OF WEIGHT IN POUNDS.		INCREMENT OF HEIGHT IN INCHES.	
	S group.	N-S group.	S group.	N-S group.
Number in the sample ..	20	19	20	19
Mean of the sample ..	1.9125	0.8421	0.6625	0.3684
Standard error of the mean ..	0.2347	0.2943	0.0619	0.0599
Difference between the two means	1.0704		0.2941	
Standard error of difference ..	0.3764		0.0860	
Difference ..	2.84		3.42	
S. E. of difference ..	Significant.		Significant.	
Significance ..	Significant.		Significant.	

TABLE III.

Ranges of variation of the mean increments in height and weight.

Attributes.	ESTIMATED RANGE OUTSIDE WHICH THE TRUE MEAN OF THE N-S GROUP MAY NOT LIE IN				EXTENT BY WHICH THE SAMPLE MEAN OF THE S GROUP EXCEEDS THE MAXIMUM RANGE OF VARIATION OF THE MEAN OF THE N-S GROUP AT	
	5 SAMPLES OUT OF 100.		1 SAMPLE OUT OF 100.		5 per cent level.	1 per cent level.
	Minimum.	Maximum.	Minimum.	Maximum.		
Weight (lb.) ..	0.2653	1.4189	0.0769	1.6073	0.4936	0.3052
Height (inches)	0.2510	0.4853	0.2127	0.5241	0.1767	0.1384

APPENDIX B.

*Experiment B.*1. *Boys.*

Analysis of the figures for initial height and weight of the experimental group of boys receiving skimmed milk (S) and the control group receiving biscuits (Bis.) reveals that the two groups did not differ significantly as regards any of the attributes. The respective statistical constants have been calculated and are compared in Table IV:—

TABLE IV.

Analysis of the statistical constants obtained from the grouping of the initial heights and weights.

	HEIGHT IN INCHES.		WEIGHT IN LB.	
	S group.	Bis. group.	S group.	Bis. group.
Individuals in the samples ..	24	24	24	24
Mean of the samples ..	42.78	44.16	37.03	40.59
S. E. of the mean	0.91	0.79	1.62	1.66
Difference between sample means		1.38		3.56
S. E. of difference		1.21		2.32
Difference				
S. E. of difference		1.14		1.53
Significance	Not significant.		Not significant.	

The increments in height and weight observed at the end of 10 weeks were greater in the case of the group receiving skimmed milk. To discover whether these differential increases could be due to chance alone the indices of significance have been worked out and are given in Table V:—

TABLE V.

Analysis of the statistical constants obtained from the height and weight increments on the two groups at the conclusion of the experiment.

	HEIGHT IN INCHES.		WEIGHT IN LB.	
	S group.	Bis. group.	S group.	Bis. group.
Mean of the samples ..	0.61	0.33	1.53	0.09
S. E. of the means	0.06	0.05	0.24	0.30
Difference between sample means		0.28		1.44
S. E. of difference		0.08		0.38
Difference				
S. E. of difference		3.41		3.75
Significance	Significant.		Significant.	

The sample means of the increment in height and weight were subjected to further analysis, and the maximum positive variation outside which the true mean of the biscuit group may not lie at 1 per cent and 5 per cent levels of significance was calculated on the theory of probability, and compared with the respective figures of the S group. The results are given in Table VI:—

TABLE VI.

Ranges of variation of the mean increments in height and weight.

		ESTIMATED RANGE OUTSIDE WHICH THE MEAN OF THE BIS. GROUP MAY NOT LIE IN		EXTENT BY WHICH THE SAMPLE MEAN OF INCREMENTS IN THE S GROUP EXCEEDS THE MAXIMUM RANGE OF VARIATION IN THE BIS. GROUP.	
		5 cases out of 100.	1 case out of 100.	5 per cent level.	1 per cent level.
Weight (lb.)	..	0.68	0.77	0.85	0.76
Height (inches)	..	0.43	0.46	0.18	0.15

From the above results it may be safely concluded on statistical grounds alone that the addition of skimmed milk caused a significant increase in height and weight in the boys receiving skimmed milk as compared to the control series receiving biscuits.

2. Girls.

The experiment was repeated on two groups of girls. The skimmed milk group (S) consisted of 16 individuals and the biscuit group (Bis.) of 18. The mean initial heights and weights of the S group were 43.80 inches and 39.63 lb., while respective figures for the Bis. group were 44.43 inches and 40.61 lb. The increments in height and weight observed at the end of 10 weeks show a differential increase (in the case of each of the attributes) in favour of the S group. In order to discover whether the enhanced increment in height and weight noted in the S group was 'real', Fisher's 't' was calculated in the case of each of the attributes, the results being shown in Table VII. This particular test was chosen since the number of individuals in each group was less than 20.

It is apparent from Table VII below that the enhanced increment in height in the case of girls receiving skimmed milk is significant, whereas the increment in weight is not significant as compared to the control group of girls receiving biscuits. In interpreting the results it should be noted that the verdict 'significant' has a definite positive value, whereas 'not significant' does not imply anything beyond 'not proved'.

TABLE VII.

Statistical data relating to height and weight increments (girls).

			INCREMENTS HEIGHT (INCHES).		INCREMENTS WEIGHT (POUNDS).	
			S group.	Bis. group.	S group.	Bis. group.
Individuals in the group	..		16	18	16	18
Mean of the sample	..		0.6719	0.3750	1.3125	0.9028
Difference between the means in favour of S group.			0.2969		0.4097	
Value of 't'	3.5716		0.9752	
Degrees of freedom	32		32	
Value of 'p'	Beyond 0.01 level.		Between 0.4 and 0.3 level.	
Significance	Significant.		Not significant.	

A DIET SURVEY OF A GROUP OF SIKH SOLDIERS.

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OWING to the paucity of information about the diet requirements of Indians, the study of the diets consumed by well-fed groups is of interest and value. The results provide a standard of comparison by means of which the diets of other groups, in which food intake is restricted by poverty, may be evaluated. The present paper describes an investigation of the diets of 20 Indian soldiers stationed in Wellington in the Nilgiri Hills, about 6,000 feet above sea-level. The inquiry lasted three weeks, and was carried out on an individual basis.

The ages of the subjects lay between 22 and 40. Sixteen of the group were on ordinary regimental duty, three were cooks, and one was a sweeper. All except the sweeper were Punjabi Sikhs. Their general health was reported to be excellent; none had been in hospital, except for minor injuries, for the previous two years. The photograph (Plate XX) gives a good idea of their general physique. The weights of the 19 Sikhs ranged between 99 lb. and 159 lb.; height between 60 inches and 70·75 inches. Weights were taken at the beginning and end of the investigation, and showed practically no alteration during the interval.

METHOD OF INVESTIGATION.

The service ration per man daily is as follows :—

Wheat flour	1·5 lb.
Pulses	3 oz.
Potatoes	2 „
Ghee	2 „
Sugar	1·5 oz.

This ration yields about 100 g. of protein, 578 g. of fat, 590 g. of carbohydrate, and has an energy value of about 3,280 calories.

Rations are issued for every 10 days from the general military stores, being then brought to the substore in the platoon barracks, from which an issue is made for each meal. We weighed each 10 days' ration as a general check on the accuracy of the individual studies. The platoon has a common mess. We visited the mess kitchen twice daily and weighed all foodstuffs before cooking, only the edible portions being weighed. In order to study individual intake, the number of 'chapattis' (unleavened whole-wheat cakes of very constant weight) consumed by each individual for 12 days was counted. The average weight of a 'chapatti' was previously determined. In the case of legumes and vegetables, it was impossible to record individual intake and this could only be deduced by dividing consumption of the whole group by the number of mouths. It is probable that this part of the ration was very evenly divided among the men.

We found that it was the habit of the soldiers to supplement the military ration by buying condensed skimmed milk, vegetables, ghee, and other foods. Intake of these extra foods was recorded on an individual basis.

In calculating the nutritive value of the diets, the figures given in Health Bulletin No. 23, 'The Nutritive Value of Indian Foods, and the Planning of Satisfactory Diets', were employed. Averages for the group are given in Table I and individual records in Table II. Table III describes the diets of five individuals in terms of actual foodstuffs; these may be taken as typical of the whole group.

TABLE I.

Average intake of calories, proximate principles, calcium, phosphorus, and iron per man daily.

Protein, g.	..	104	Calcium, g.	..	0.71
Fat, g.	..	68	Phosphorus, g.	..	2.5
Carbohydrate, g.	..	634	Iron, mg.	..	44
Calories	3,565

PLATE XX.



Two members of the group of Sikh soldiers investigated.

TABLE II.

Individual intake.

Number.	Weight		Height, inches.	Occupation.	Protein, g.	Fat, g.	Carbohydrate, g.	Calo- ries.	Calcium, g.	Phos- phorus, g.	Iron, mg.	Vitamin A, Inter- national Units.	Carotene, Inter- national vitamin A Units.	Total vitamin A and carotene, in Inter- national Units.
	Before investi- gation, lb.	After investi- gation, lb.												
1	143	143	70-75	Ordinary regimental duty.	116.8	92	680	3,734	0.90	2.83	48	2,293	1,390	3,683
2	118	123	66-25	"	107.3	56	678	3,660	0.88	2.68	45	1,312	1,115	2,427
3	125	126	64-00	"	109.8	64	676	3,621	0.79	2.62	46	1,478	1,719	3,197
4	116	116	63-50	"	109.4	57	667	3,669	0.92	2.71	45	1,343	1,110	2,453
5	112	112	63-25	"	102.0	94	692	3,603	0.92	1.90	42	2,363	1,264	3,627
6	100	102	61-00	"	106.1	50	629	3,407	0.64	2.50	45	1,071	2,143	3,214
7	140	140	68-00	"	99.7	104	597	3,769	0.72	2.47	43	2,814	950	3,764
8	120	132	67-75	"	92.3	56	635	3,466	0.60	2.63	46	1,183	2,116	3,299
9	159	161	68-00	"	109.5	56	686	3,678	0.89	2.68	45	1,312	1,320	2,632
10	126	127	66-00	"	111.7	72	685	3,855	0.69	2.65	46	1,644	1,044	2,688
11	142	144	67-50	"	108.2	59	663	3,669	0.66	2.67	46	1,246	1,537	2,783
12	146	148	67-75	"	94.9	95	640	3,883	0.64	2.41	43	2,491	1,564	4,055
13	121	126	64-75	"	106.0	69	650	3,556	0.63	2.74	45	1,629	1,042	2,671
14	146	150	69-50	"	111.8	79	655	3,871	0.68	2.64	46	1,749	1,298	3,047
15	118	121	65-75	"	99.0	83	637	3,608	0.61	3.02	42	2,090	1,263	3,353
16	126	126	68-00	"	96.6	91	573	3,539	0.57	1.73	39	2,393	955	3,348
17	99	102	60-00	Cook	106.6	50	605	3,351	0.62	2.50	44	1,103	943	2,046
18	116	119	66-00	"	107.1	48	640	3,441	0.65	2.54	46	1,060	1,846	2,906
19	111	112	61-50	"	101.2	46	592	3,198	0.59	2.42	44	1,833	959	2,792
20	95	96	60-00	Sweeper	85.0	44	493	2,721	0.52	1.92	36	1,033	809	1,842

TABLE III.

Composition of the diet of various individuals (grammes daily).

	Subject No. 1.	Subject No. 7.	Subject No. 11.	Subject No. 12.	Subject No. 14.
Whole wheat flour ..	519	610	642	603	628
Rice	40	40	45	48	45
Pulses	80	80	80	80	80
Leafy vegetables ..	14	7	7	7	7
Potatoes	67	54	64	54	56
Other vegetables ..	139	99	93	106	103
Fruits	16	48	93	69
Meat	42	13	30	26	28
Milk (whole)	33	35	33	33	33
Milk (skimmed and sweetened)	31	16	3	3	3
Ghee	92	110	46	96	65
Sugar	84	60	51	71	58

The items grouped under a common head in the above table were as follows :—

Pulses—Bengal gram, black gram, green gram, and dhal arhar.

Leafy vegetables—Cabbage, coriander leaves, and fenugreek.

Other vegetables—Radish, turnip, beetroot, carrot, onion, cauliflower, brinjal, vegetable marrow, pea, knol-khol, tomato, and bitter gourd.

Fruits—Plantain, mango, orange, and hill guava.

Meat—Mutton and fowl.

DISCUSSION.

Intake of calories was high. It is to be observed that the soldiers were strenuously employed. The barracks are situated on an elevation of some 120 feet above the mule stables, which the men visited some five to seven times a day. Their ordinary regimental duties included parades, route marches, the care of the mules, etc., and in addition they indulged in sports. The high calorie intake is in contrast to that observed in the case of poor South Indian village families, which is usually considerably less than 2,500 calories per 'consumption unit' or 'adult man' daily. The soldiers were living in the comparatively cool climate of the Nilgiris, and more energy is required for heat production in the hills than in the plains. Nevertheless diet surveys of tea plantation coolies living at the same altitude have revealed an average calorie intake which is about two-thirds of that of the military group. The present investigation shows that a group of active Indians will consume as much food as a group of active Europeans, and it is probable that the low calorie intake of Indians of the poorer classes is due to want rather than to race or climate.

Protein.—The average daily intake of protein exceeded 100 grammes. It was in excess of that suggested by Sherman (1933) and the League of Nations Technical Commission on Nutrition (1936) which was one gramme per kg. of body-weight. Very little animal protein was included in the diet; intake was in the neighbourhood of 6 to 7 grammes daily. The percentage of total protein intake supplied by animal protein was therefore about 7 grammes, which is lower than that recommended in the standard textbooks.

Fat.—Fat intake was adequate, and of the total average consumption, which reached 68 g., 54 g. were animal fat.

Mineral salts.—The intake of phosphorus, calcium, and iron exceeded textbook standards.

Vitamins.—The diet was rich in vitamin A and carotene, the former being supplied by ghee. At a rough estimate intake of International Units of vitamin A was about 3,000 per day. This corresponds with the intake usually recommended as satisfactory.

Owing to its high content of whole wheat, the diet was rich in the B group of vitamins. Average daily intake of vitamin B₁ exceeded 1,500 International Units. Vitamin C was also present in sufficient amount.

Hæmoglobin.—The hæmoglobin content of the blood was investigated by the colorimetric method described by Sankaran and Rajagopal (1938), and the results are stated in terms of grammes of hæmoglobin per 100 c.c. of blood. The figures are as follows:—

TABLE IV.

Hæmoglobin per 100 c.c. of blood.

Number.	Amount of hæmoglobin present in 100 c.c. of blood.	Number.	Amount of hæmoglobin present in 100 c.c. of blood.
1 ..	17.51	11 ..	21.61
2 ..	18.88	12 ..	20.01
3 ..	24.20	13 ..	19.43
4 ..	21.61	14 ..	19.81
5 ..	17.97	15 ..	23.11
6 ..	21.61	16 ..	17.81
7 ..	21.85	17 ..	23.23
8 ..	21.61	18 ..	24.64
9 ..	20.62	19 ..	21.06
10 ..	19.91		

These figures are very much in excess of textbook averages, which may be ascribed to the fact that the subjects were living at a high altitude.

Blood pressure.—The systolic blood pressure of the individuals forming the group ranged between 110 mm. and 122 mm., and the diastolic between 60 mm. and 80 mm. These figures are within the normal limits.

SUMMARY.

(1) A diet survey of healthy Sikh soldiers has been carried out. The diet was found to have a high energy content and was adequate in respect of total protein, fat, vitamins, and mineral salts.

(2) The group showed a high hæmoglobin figure, presumably due to altitude. Systolic and diastolic blood pressures were within normal limits.

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STUDIES ON VITAMIN-A DEFICIENCY.

Part III.

LESIONS OF THE PERIPHERAL NERVOUS SYSTEM.

BY

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APART from xerophthalmia, several workers have described lesions of the nervous system, shown by degeneration of the myelin sheaths, in experimental animals subsisting on diets low in carotene or vitamin A. Zimmerman (1933), and Zimmerman and Cowgill (1936) described muscular weakness, inco-ordination, and, in severe cases, paralysis of the hind legs in albino rats fed on vitamin-A deficient diets. These workers observed degeneration of the medullary sheaths of the brachial plexus, the sciatic nerves and the sensory tracts on the periphery of the spinal cord and in the posterior columns. Hughes, Lienhardt and Aubel (1929) reported that in pigs, chickens and cows, lack of vitamin A in the diet resulted in marked nervous disorders, characterized by impaired vision, inco-ordination and spasms. Histological examination revealed degeneration of the nerve bundles in the optic, sciatic, and femoral nerves and scattered degeneration in the spinal cord. Similar symptoms and histological evidence of nerve degeneration in swine were observed by Hart, Miller and McCollum (1916), Wehrbein (1916), and Biester and Murray (1933). Hart and his co-workers (*loc. cit.*) ascribed the degenerative changes in the nervous system to a toxic factor in the wheat kernel, although the rations employed by these workers were low in vitamin A and the addition of alfalfa or commercial meat scraps prevented the occurrence of nervous symptoms. Mellanby (1926 to 1935) described lesions of the central and peripheral nervous systems produced in young puppies and rabbits by diets containing a large amount of cereal (other than yellow maize) and deficient in vitamin A and carotene. Degenerative changes were most marked in the ascending fibres—both exogenous and endogenous—in the central nervous system, and the afferent fibres in the peripheral nervous system. Nervous disorders accompanying avitaminosis A have also been described by Steenbock, Nelson and Hart (1921-22) in the dog, by Elvehjem and Neu (1932) and Seifried (1932) in chicks, and by Kingery and Kingery (1925), Aberle (1934), and Duncan (1930) in albino rats.

Most of the above-mentioned workers used the Marchi and other osmic acid techniques for the detection of degenerative changes in the nervous system. Recently, Sutton, Setterfield and Krauss (1934), and Setterfield and Sutton (1935) have applied the polarized light technique in the study of degeneration of myelinated nerves occurring in vitamin-A deficiency in white rats. Their findings are in agreement with those described by the other workers mentioned above.

Opinion, however, is not unanimous regarding the relation of vitamin-A deficiency to changes in the nervous system. Conflicting results have been obtained by other workers. Wolbach and Howe (1925), who made an exhaustive study of the tissue changes following deprivation of vitamin A in the white rat, found no degenerative lesions in the brain, cerebellum, sympathetic ganglia, and ganglionic cells of the myenteric plexus. An investigation of the peripheral nerves was not made by these workers, as the animals showed no symptoms of nervous disorder. Suzman, Muller and Ungley (1932) found no degenerative changes in the spinal cord of dogs fed on a high cereal diet deficient in vitamin A. Stockman and Johnston (1933), and Elder (1935) found demyelination of the nerves in animals fed on diets adequate in vitamin A. No significant changes in the central nervous system were noticed by Grinker and Kandel (1933), Weil and Davison (both quoted by Sweet and K'Ang, 1935), in experimental vitamin deficiency in monkeys and rats. More recently Eveleth and Biester (1937) have shown that myelin degeneration of the spinal cord and peripheral nerves in swine may be caused by dietary factors other than deficiency of vitamins A and B. They observed severe myelin degeneration in dogs fed on a diet which contained vitamins A, B complex, D, and E.

Several workers have described lesions of the peripheral nerves in animals fed on a diet deficient in the vitamin-B complex (McCarrison, 1921 ; Cowgill, 1921 ; Stern and Findlay, 1929 ; Zimmerman and Burack, 1932 ; Gildea, Castle, Gildea and Cobb, 1935 ; Lee and Sure, 1937 ; and others). Stockman and Johnston (*loc. cit.*), who produced nerve lesions in monkeys by feeding a high cereal diet adequately supplemented by vitamin A, ascribed the degeneration to toxic substances contained in cereals. Mellanby (*loc. cit.*) similarly attributed the lesions of the nervous system produced in animals by diets containing a large amount of cereals, and deficient in vitamin A or carotene, to cereal toxins. Nervous lesions have also been ascribed to a deficiency of calcium (Corlette, 1928, 1929) and vitamin E (Evans and Burr, 1928).

Wolbach (1937), in a recent contribution dealing with the pathologic changes resulting from vitamin deficiency, maintains that further work on the subject is necessary to establish a specific relation between vitamin-A deficiency and myelin degeneration, since the latter is common to so many disorders of man and animals and may be the result of several vitamin deficiencies and diverse causes. For the present, Wolbach (*loc. cit.*) regards nervous lesions as being among the late secondary consequences of vitamin-A deficiency.

There is thus some confliction of evidence regarding the relation between vitamin-A deficiency and nerve lesions. The present paper reports a systematic investigation of the peripheral nerves in vitamin-A deficient animals, which was undertaken to throw light on the problem. The subject is one of practical importance on account of the frequency with which clinical conditions associated with vitamin-A deficiency occur in South India.

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PLATE XXI.

[The photomicrographs were taken with 'Miftex' (Zeiss).]

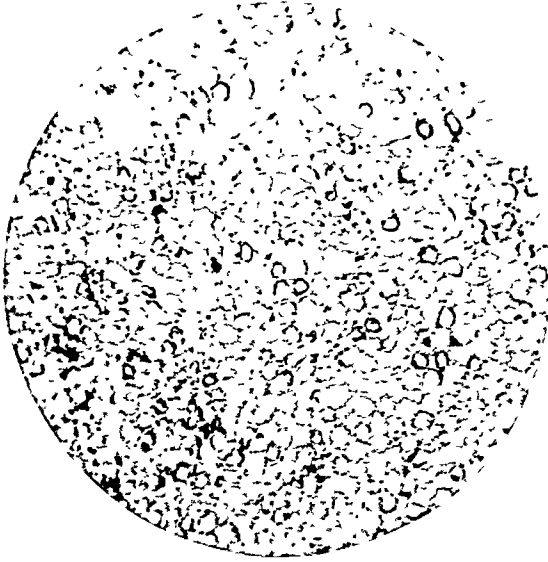


Fig. 1.—*Rabbit No. 1*.—Sciatic nerve showing 'annular degeneration'; Marchi's stain. $\times 200$.

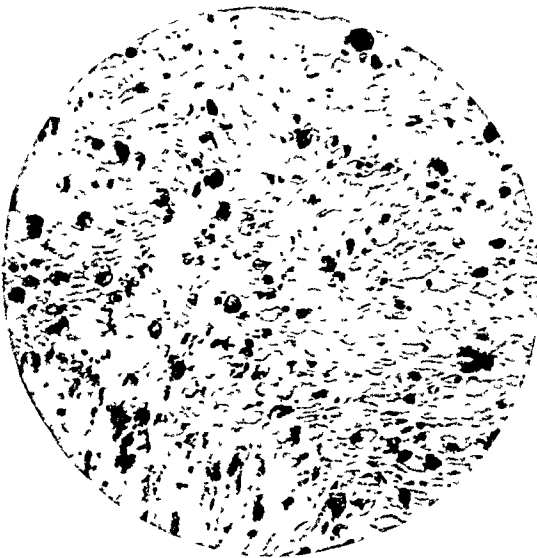


Fig. 2.—*Fowl No. 4*.—Femoral nerve showing fine and coarse granules of disintegrated myelin; Marchi's stain. $\times 200$. (This bird developed leg weakness.)

TABLE I.
Nerve lesions in rabbits fed on a vitamin-A deficient diet.

Serial number.	Duration of experiment (days).	Condition of the eye*.	LESIONS OF THE PERIPHERAL NERVES (MICROSCOPIC DIAGNOSIS)†.						Antimony trichloride test for vitamin A in the liver. — negative. + positive.	REMARKS.
			Trigeminal.	Optic.	Sciatic.	Femoral.	Median.	Ulnar.	Radial.	
1	136	a	3	3	3	3	3	Parasitic infection of the liver; abscesses in the upper lobe of right lung.
2	135	a	1	1	1	1	1	Parasitic infection of the liver. Xerophthalmia treated first with red palm oil; eye signs developed again after stopping treatment.
3	78	c	1	1	1	
4	85	c	1	1	1	
5	126	c	1	1	1	1	1	Broncho-pneumonia of right lung. Broncho-pneumonia of right lung.
6	141	o	1	1	1	1	1	
7	191	c	1	1	1	1	1	
8	151	c	1	1	1	1	1	(faint).
9	148	c	1	1	1	1	1	
10	154	c	1	1	1	1	1	
11	169	d	..	1	1	1	1	..	1	(faint).
12	82	d	1	1	1	1	1	2	1	
13	107	d	2	3	2	1	1	1	1	
14	125	d	1	1	1	1	1	(faint).
15	148	d	1	1	1	1	1	
16	136	d	1	1	1	1	1	

* a = No eye changes, macroscopic or microscopic.

b = Sore eyes (shown by photophobia and increased secretion), but no demonstrable affection of the cornea and no xerosis of the conjunctiva.

c = Clouded cornea; xerosis of the cornea and/or of the conjunctiva on one or both sides.

d = Corneal ulceration in several stages of development.

† 0 = No degeneration. 1 = Slight degeneration.

2 = Moderate degeneration.

3 = Marked degeneration.

TABLE II.

Nerve lesions in albino rats fed on a vitamin-A deficient diet.

Serial number.	Duration of experiment (days).	Condition of the eye*.	LESIONS OF THE PERIPHERAL NERVES (MICROSCOPIC DIAGNOSIS)†.						Antimony trichloride test for vitamin A in the liver. - negative. + positive.	REMARKS.
			Trigeminal.	Optic.	Sciatic.	Femoral.	Median.	Ulnar.	Radial.	
1	270	a	0	0	0	0	0	+
2	270	a	0	0	0	0	0	-
3	270	a	1	1	1	1	1	-
4	270	a	0	0	0	0	0	+
5	149	a	1	0	1	1	1	-
6	119	c	3	..	1	..	1	-
7	145	d	1	..	1	0	1	-
8	51	c	1	1	0	0	0	-
9	87	c	2	2	0	0	1	-
10	87	c	2	2	1	1	1	-

Small ulcer in the left sub-maxillary region.

Abscess at the base of the tongue.

* and †: See notes under Table I.

TABLE III.

Nerve lesions in fowls fed on a vitamin-A deficient diet.

Serial number.	Duration of experiment (days).	Condition of the eye*.	LESIONS OF THE PERIPHERAL NERVES (MICROSCOPIO DIAGNOSIS)†.						Antimony trichloride test for vitamin A in the liver. - negative. + positive.	REMARKS.
			Trigeminal.	Optic.	Sciatic.	Femoral.	Median.	Ulnar.	Radial.	
1	38	a	2	2	2	1	+ (7 per gramme of fresh liver). + (21 per gramme of fresh liver).
2	71	a	2	2	1	1	1	..	1	
3	226	a	1	1	1	1	1	-
4	75	c	3	2	-
5	42	c	1	2	1	0	1	..	1	-
6	122	c	1	1	1	1	1	..	1	-
7	62	d	1	1	1	1	1	1	1	-

Paresis of the legs.

Animal looking sick; sitting in a hunched position.

* and †: See notes under Table I.

GROUP IV: *Seven fowls on diets deficient in carotene and vitamin A.*—Slight or moderate degree of myelin degeneration was seen in three birds, in which evidences of xerophthalmia were not present (*vide* Table III). One bird, which developed paresis of the legs and xerophthalmia, showed moderate degeneration in the femoral nerves. The rest showed slight degeneration of the myelin sheaths in the peripheral nerves. As in the rabbits, the degree of myelin degeneration in the nerves of the birds was not proportional to the intensity of the xerophthalmia.

DISCUSSION.

The present investigation shows that demyelination of the peripheral nerves occurs in animals of various species fed on diets deficient in vitamin A. Most of those who have studied the pathology of vitamin-A deficiency have confined their attention to the changes occurring in epithelial tissues, and the nerve lesions have not received sufficient attention. Though there was variation in the degree of myelin degeneration, the peripheral nerves in the animals in groups I, III, and IV in this series showed consistent changes. The albino rats in group II were obtained, as already mentioned, from stock animals which received a diet rich in vitamin A and carotene and hence these animals on weaning had considerable quantities of vitamin A stored in the liver. This high storage of vitamin A in the liver appears to be the cause of the low incidence of xerophthalmia and the comparatively slight myelin degeneration in the peripheral nerves found in the animals in this group (group II).

In most instances, all the spinal nerves examined showed about the same degree of myelin degeneration, and there appeared to be no selective involvement of any of the nerves. In some animals, however, the sciatic and femoral nerves showed more marked degenerative changes than those present in the brachial plexuses.

The xerophthalmia occurring in the animals in this series can unquestionably be attributed to vitamin-A deficiency, and it seems reasonable to ascribe the myelin degeneration to the same cause. There was no exact parallelism, however, between the intensity of the eye lesions and the degree of demyelination of the peripheral nerves. In some instances, nerve lesions were found in animals which showed no xerophthalmia. Sutton *et al.* (*loc. cit.*) and Setterfield and Sutton (*loc. cit.*) demonstrated degenerative changes in the sciatic and femoral nerves before the appearance of the earliest signs of avitaminosis A. The progress of the nerve degeneration appears to be slow and does not keep pace with the development of xerophthalmia. The degenerative changes were only slight or moderate even in animals showing marked eye lesions. As pointed out in a previous paper (Radhakrishna Rao, *loc. cit.*) the general condition of the animal, and the length of time during which the deficient diet is consumed, are probably among the factors influencing the degree of demyelination in the nerves. The variations in the lesions may also be due to the age of the animal at the commencement of the experiment and the food it receives before and after birth.

Except one bird, which developed paresis of the legs, none of the animals showed any definite clinical evidence of involvement of the peripheral nerves. Setterfield and Sutton (*loc. cit.*) reported that in rats suffering from avitaminosis

A, between one-fourth and one-third of the fibres in the sciatic and femoral nerves were in the process of degeneration before there was evidence of loss of sensibility or motor control. Demyelination of the peripheral nerves may thus be present without the occurrence of clinical symptoms. More extensive lesions than those seen in the present series of animals are probably necessary to produce paralytic symptoms.

A considerable series of observations, including those reported here, point to the conclusion that vitamin A is related to the integrity of the nervous tissues, but at the same time it appears that myelin degeneration may be associated with various forms of dietary error.

We have at present no definite knowledge regarding the rôle of vitamin A and carotene deficiency in nervous disease in man. Mellanby (1931, 1933, 1934a) is of opinion that the vitamin-A and carotene deficiency may play a part in the causation of certain disorders, e.g., convulsive ergotism, lathyrism, sub-acute combined degeneration and disseminated sclerosis. Nervous diseases appear to be fairly common in South Indians, and it would be interesting to study the effect of vitamin A and carotene in their treatment. Iswariah and Kutumbiah (1934) have described a variety of polyneuritis occurring in women during the puerperium in Northern Circars. Nicholls (1935) has made reference to the occurrence of peripheral neuritis in women in the later stages of pregnancy and during lactation in Ceylon. Cases similar to those described by the above authors are common in lactating women in South India. The evidence available at present suggests that a lack of vitamin B₁ in the food is the predominant factor in the causation of polyneuritis in these cases, but vitamin-A deficiency may perhaps also play a part. This problem remains to be investigated.

SUMMARY.

(1) The peripheral nervous system was studied in rabbits, albino rats, and fowls fed on diets deficient in vitamin A and carotene. Clinically, evidences of nervous disorder were rarely observed. Histological examination, however, revealed varying degrees of degeneration of the myelin sheaths of the spinal nerves.

(2) It is possible that, while there is good evidence to show that vitamin-A deficiency causes myelin degeneration, similar changes may result from other dietetic factors.

(3) The relation between dietary deficiency and certain nervous diseases in man is discussed.

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THE OXALIC-ACID CONTENT OF SOME INDIAN FOODSTUFFS.

BY

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No data appear to exist about the oxalic-acid content of common Indian foodstuffs. The receipt in the Laboratories of inquiries from a number of medical men drew our attention to this fact. It was therefore thought useful to carry out a series of oxalic-acid determinations on foodstuffs, as part of the study of the composition of Indian foodstuffs now in progress in these Laboratories. The possibility of such a survey throwing light on the problem of urinary calculus in India was also considered.

METHOD OF ESTIMATION.

For the estimation of oxalic acid the following method, representing a modification of the standard chemical method, was worked out: 10 to 20 grammes of the fresh test substance were ground with glass powder in a mortar, and the whole material was transferred into a beaker. It was then boiled with 15 per cent hydrochloric acid, filtered over a Buchner, and thoroughly washed. The filtrate was made alkaline with ammonia, re-acidified with acetic acid, and finally precipitated hot with CaCl_2 solution. This was filtered, the residue boiled with acetic acid and again filtered, after which it was thoroughly washed with water to make it free from chloride. The filter-paper was ignited and ashed in the electric furnace. The CaO was next dissolved in a little hydrochloric acid, made alkaline with ammonia, and precipitated with ammonium oxalate solution. The calcium oxalate thus formed was filtered and washed free from the soluble oxalate, and titrated with potassium permanganate in the usual way.

The Arbenz (1917) method was also tried, but it did not prove a success in our hands, and it seemed to be more complicated and time-consuming than the

method adopted. The present method appears to give consistent results. The recovery from solutions to which known amounts of oxalic acid had been added was practically cent per cent. The oxalic-acid values given by Nelson and Motter (1931), Ryder (1930), and Esbach (1883) agree fairly well with ours.

The foodstuffs analysed were bought in the local market in Coonoor, in as fresh a state as possible. Unless otherwise stated, each figure represents the average of two determinations.

TABLE.

The oxalic-acid content of certain Indian foodstuffs.

Name of foodstuff.	Botanical name.	Mg. per 100 g.
A. Cereals and pulses.—		
Rice	<i>Oryza sativa</i>	4.6
Wheat	<i>Triticum vulgare</i>	11.1
Cholam	<i>Sorghum vulgare</i>	12.6
Cambu or bajra	<i>Pennisetum typhoideum</i>	14.4
Ragi	<i>Eleusine coracana</i>	45.7
Maize	<i>Zea mays</i>	9.0
Tapioca	<i>Manihot utilisima</i>	17.1
Bengal gram	<i>Cicer arietinum</i>	2.4
Black gram	<i>Phaseolus mungo</i>	27.8
Lentil	<i>Lens esculenta</i>	21.2
Soya bean	<i>Glycine hispida</i>	77.0
B. Vegetables.—		
Lettuce	<i>Lactuca sativa</i>	13.6
Cabbage	<i>Brassica oleracea capitata</i> (4 samples).	5.9–18.5
Spinach	<i>Spinacia oleracea</i>	658.0
Potato	<i>Solanum tuberosum</i>	15.0
Colocasia	<i>Colocasia antiquorum</i>	133.4
Green plantain	<i>Musa paradisiaca</i> (3 samples)	517.5–524.0
Carrot	<i>Daucus carota</i>	5.6

TABLE—contd.

Name of foodstuff.	Botanical name.	Mg. per 100 g.
B. Vegetables—concl'd.		
Brinjal	<i>Solanum melongena</i>	29.1
Bitter gourd	<i>Momordica charantia</i>	0.5
Snake gourd	<i>Trichosanthes anguina</i>	1.0
Coriander leaves	<i>Coriandrum sativum</i>	5.0
Mint	<i>Mentha viridis</i>	3.8
Onion	<i>Allium cepa</i>	1.0
Cauliflower	<i>Brassica oleracea botrytes</i>	6.8
Green chillies	<i>Capsicum annum</i>	25.7
Radish	<i>Raphanus sativus</i>	9.2
Ash gourd	<i>Benincasa cerifera</i>	3.4
French beans	<i>Phaseolus vulgaris</i>	31.2
Beetroot	<i>Beta vulgaris</i>	40.4
Parsnip	<i>Pastinaca sativa</i>	20.5
Green peas	<i>Pisum sativum</i>	6.0
Lady's finger	<i>Hibiscus esculentus</i>	10.3
Tomato (ripe)	<i>Lycopersicum esculentum</i>	3.6
Rhubarb (stalk)	<i>Rheum rhaponticum</i>	1336.0
Mustard leaves	<i>Brassica juncea</i>	128.7
C. Fruits.—		
Plantain (ripe)	<i>Musa paradisiaca</i>	2.2
Grape fruit (juice)	<i>Vitis vinifera</i>	3.4
Oranges	<i>Citrus aurantium</i>	8.7
Ripe mango	<i>Mangifera indica</i>	30.0
Pine-apple	<i>Ananas sativus</i>	5.8
Almonds	<i>Prunus amygdalis</i>	407.3
Cashew-nut	<i>Anacardium occidentale</i>	318.4

TABLE—concl'd.

Name of foodstuff.	Botanical name.	Mg. per 100 g.
D. <i>Animal foods.</i> —		
Mutton	6.9
Sheep's liver	4.2
„ brain	4.6
Beef	25.0
Cow's milk	1.9
E. <i>Miscellaneous.</i> —		
Ripe chillies	<i>Capsicum annum</i>	117.1
Cocoa (Rowntree's)	442.0*
Coffee powder	15.4†
Tea (Lipton's—dust)	219.2‡

* Cocoa powder itself was extracted.

† Powder itself was extracted.

‡ Ten grammes dust lixiviated in 200 c.c. of boiling water for five minutes and the total filtrate taken for estimation.

From the Table it will be seen that amongst the vegetables, rhubarb stands first in oxalic-acid content. Next comes spinach. Amongst cereals and pulses, ragi, and amongst the fruits, almond and cashew-nut contain the largest amounts. Animal foods seem to contain very little, which is also true of ordinary fruits. The oxalic-acid content of cocoa was found to be high, approximating to that of spinach. Milk also contains oxalic acid, though in almost negligible amounts.

It is evident that before any attempt can be made to relate the incidence of stone and the oxalic-acid content of the diet, more information must be obtained about the diets consumed in areas of the country in which urinary calculus is common. Attention may be drawn to one interesting fact: there is possibly

a relation between the oxalic-acid content of a foodstuff and the degree to which the calcium and magnesium it contains are retained in the system. Ranganathan (1935) gave the following figures representing calcium and magnesium retention in rats fed exclusively on various cereals:—

		Ca, per cent.	Mg., per cent.
Rice	..	87.1	62.1
Wheat	..	73.5	50.3
Cholam	..	70.4	..
Carabu	..	49.5	..
Ragi	..	42.6	3.8

Rice, which gave the highest figures for the retention of calcium and magnesium, has the lowest oxalic-acid content of all the cereals investigated, while ragi, with the lowest calcium and magnesium retention figures, has the highest oxalic-acid content. It seems possible that the oxalic-acid content of a foodstuff may be a factor influencing the retention of the calcium and magnesium it contains.

SUMMARY.

Fifty-two common foodstuffs including cereals and pulses, vegetables, fruits, animal foods, and beverages have been analysed for their oxalic-acid content by a modified method.

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THE 'AVAILABLE' IRON IN SOME COMMON INDIAN FOODSTUFFS, DETERMINED BY THE α, α' DIPYRIDINE METHOD.

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THE term 'available' iron was first used by Hill (1930) and later by Elvehjem and his co-workers (1933, 1934, 1936) to denote that fraction of iron in foodstuffs which is capable of combining with α, α' dipyridine. The latter workers have shown that the efficiency of a food in regenerating hæmoglobin in anæmic rats is correlated, not with its total iron content, but rather with the fraction combining with α, α' dipyridine. Hence this fraction of iron, held in simple combination, was termed 'available' iron in contrast to the gross total iron, not all of which is available for use in fulfilling the physiological function of iron. The total iron content of foodstuffs is, therefore, of relatively little value in nutritional studies, unless accompanied by information with regard to the percentage 'availability' of iron in each instance. Elvehjem and his collaborators have described a method for estimating chemically the 'available' iron in foodstuffs, and have shown that the results obtained by this method agree closely with those yielded by time-consuming tests on rats. In the present work, this method, slightly modified, has been applied to a number of foodstuffs in common use.

EXPERIMENTAL.

The method employed makes use of α, α' dipyridine, a reagent first used by Hill (*loc. cit.*), which selectively reacts with the less complex 'available' iron moiety of foodstuffs. The optimum conditions for this reaction have been worked out by Elvehjem and his co-workers and reported in their latest paper (Kohler, Elvehjem and Hart, 1936). The substitution of hydroquinone for sodium hydrosulphite, for reducing the iron present in foodstuffs to the bivalent condition, in which state alone it reacts with dipyridine, is a distinct improvement as the former can more easily be purified and rendered iron-free. However, even the method evolved finally had certain drawbacks, including the not infrequent production of a turbid centrifugate which is difficult to use for colorimetric comparison, and the difficulty

TABLE—contd.

Name of foodstuff.			Botanical name.	Total iron, mg. per cent.	Available iron, mg. per cent.	Per cent of total iron available.
<i>Pulses—concl'd.</i>						
Green gram	<i>Phaseolus radiatus</i>	10.00	1.26	12.6
Lentil	<i>Lens esculenta</i>	1.98	1.73	87.5
Peas, roasted	<i>Pisum sativum</i>	5.00	2.48	49.6
Red gram	<i>Cajanus indicus</i>	5.56	1.30	23.3
Soya bean	<i>Glycine hispida</i>	9.70	2.76	28.5
<i>Leafy vegetables.—</i>						
' Agathi '	<i>Sesbania grandiflora</i>	3.91	0.75	19.2
Amaranth, tender leaves	<i>Amaranthus gangeticus</i>	23.70	6.00	25.3
Cabbage	<i>Brassica oleracea capitata</i>	0.73	0.28	38.3
Coriander, tender leaves	<i>Coriandrum sativum</i>	9.97	1.08	10.9
Curry leaves	<i>Murraya Koenigii</i>	8.70	4.35	50.0
Drumstick, tender leaves	<i>Moringa oleifera</i>	7.00	1.67	23.9
Fenugreek leaves..	<i>Trigonella foenumgraecum</i>	16.90	2.69	16.0
Lettuce	<i>Lactuca sativa</i>	2.39	1.00	42.1
Mint	<i>Mentha viridis</i>	15.56	1.44	9.3
Parsley	<i>Petroselinum sativum</i>	17.86	3.18	17.8
' Manathakkali '	<i>Solanum nigrum</i>	15.30	2.48	16.2
Spinach	<i>Spinacia oleracea</i>	5.00	0.93	18.6
<i>Roots and tubers.—</i>						
Beetroot	<i>Beta vulgaris</i>	0.98	0.53	53.2
Carrot	<i>Daucus carota</i>	2.83	0.26	9.2
Colocasia	<i>Colocasia antiquorum</i>	1.20	0.33	27.5
Parsnip	<i>Pastinaca sativa</i>	0.54	0.46	85.3
Potato	<i>Solanum tuberosum</i>	0.76	0.34	44.8
Radish (pink)	<i>Raphanus sativus</i>	0.47	0.11	23.5
Tapioca	<i>Manihot utilissima</i>	0.92	0.41	44.6
Yam, elephant	<i>Amorphophallus campanulatus</i>	0.62	0.39	62.9

TABLE—*contd.*

Name of foodstuff.	Botanical name.	Total iron, mg. per cent.	Available iron, mg. per cent.	Per cent of total iron available.
<i>Other vegetables.—</i>				
Amaranth, tender stalks ..	<i>Amaranthus gangeticus</i>	3.29	1.36	41.4
Ash gourd	<i>Benincasa cerifera</i>	1.06	0.60	56.7
Bitter gourd	<i>Momordica charantia</i>	1.34	0.27	20.2
Brinjal	<i>Solanum melongena</i>	9.44	0.37	84.1
Broad beans	<i>Dolichos lablab</i> var. <i>lignosus</i>	1.61	0.61	37.9
Cauliflower	<i>Brassica oleracea botrytes</i>	1.38	0.41	29.8
Cluster beans	<i>Cyamopsis psoralioides</i>	3.13	0.80	25.6
Double beans	<i>Faba vulgaris</i>	2.27	1.57	69.4
Drumstick	<i>Moringa oleifera</i>	5.28	0.48	9.1
French beans	<i>Phaseolus vulgaris</i>	1.67	1.03	61.7
Jack-fruit seeds ..	<i>Artocarpus integrifolia</i>	1.67	0.56	33.5
Lady's fingers	<i>Hibiscus esculentus</i>	1.54	0.55	35.7
Peas, English (green) ..	<i>Pisum sativum</i>	1.90	1.48	78.0
Plantain, green	<i>Musa paradisiaca</i>	0.71	0.05	7.1
Snake-gourd	<i>Trichosanthes anguina</i>	0.88	0.22	25.0
Spinach, stalks	<i>Spinacia oleracea</i>	1.90	0.72	37.9
'Sandakai', dry	<i>Solanum torvum</i>	22.22	1.52	6.9
<i>Nuts and oilseeds.—</i>				
Almond	<i>Prunus amygdalis</i>	5.56	2.37	42.7
Cashew-nut	<i>Anacardium occidentale</i>	4.95	1.94	39.1
Coco-nut, kernel	<i>Cocos nucifera</i>	1.94	0.75	38.8
Ground-nut	<i>Arachis hypogea</i>	1.63	0.50	30.7
Linseed	<i>Linum usitatissimum</i>	2.65	0.98	37.0
Mustard	<i>Brassica juncea</i>	17.88	2.27	12.7

TABLE—*contd.*

Name of foodstuff.	Botanical name.	Total iron, mg. per cent.	Available iron, mg. per cent.	Per cent of total iron available.
<i>Condiments, spices, etc.—</i>				
' Arisithippili '	<i>Piper elusii</i>	13.51	3.14	23.3
Cardamom	<i>Elettaria cardamomum</i>	5.18	0.58	10.6
Chillies, green (ordinary variety).	<i>Capsicum annuum</i>	1.31	0.41	31.4
Chillies, dry	2.25	1.34	59.7
.. giant	<i>Capsicum frutescens</i>	1.04	0.83	80.0
Coriander seeds	<i>Coriandrum sativum</i>	17.24	2.14	12.0
Fenugreek seeds	<i>Trigonella foenumgræcum</i>	14.10	1.46	10.4
Garlic	<i>Allium sativum</i>	1.31	0.72	55.0
Ginger	<i>Zingiber officinale</i>	2.57	0.57	22.2
Mustard	<i>Brassica juncea</i>	17.88	2.27	12.7
Nutmeg	<i>Myristica fragrans</i>	4.57	0.73	16.0
' Onum '	<i>Carum copticum</i>	14.62	2.74	18.7
Onion, small	<i>Allium cepa</i>	1.61	1.14	71.0
Pepper, dry	<i>Piper nigrum</i>	16.80	3.24	19.3
Tamarind (pulp only) ..	<i>Tamarindus indicus</i>	10.90	2.42	22.2
Turmeric	<i>Curcuma longa</i>	18.60	1.81	9.7
<i>Fruits.—</i>				
Apple	<i>Pyrus malus</i>	0.73	0.21	28.8
Cape gooseberry	<i>Physalis peruviana</i>	1.07	0.29	27.2
Currants	<i>Ribes</i> spp.	8.99	1.23	14.4
Lime (juice only)	<i>Citrus medica</i> var. <i>acida</i>	2.20	1.14	51.9
Mangosteen	<i>Garcinia mangostana</i>	0.58	0.23	39.7
Orange	<i>Citrus aurantium</i>	0.53	0.22	41.5
Passion fruit	<i>Passiflora laurifolia</i>	1.27	0.32	25.2
Pears, country	<i>Pyrus communis</i>	0.68	0.40	59.0

TABLE—concl'd.

Name of foodstuff.	Botanical name.	Total iron, mg. per cent.	Available iron, mg. per cent.	Per cent of total iron available.
<i>Fruits—concl'd.</i>				
Pineapple	<i>Ananas sativus</i>	1.50	1.06	70.7
Plantain, ripe	<i>Musa paradisiaca</i>	0.66	0.31	47.0
Pomelo	<i>Citrus decumana</i>	0.46	0.10	21.8
Tree tomato	<i>Cyphomandra betacea</i>	0.88	0.50	56.9
<i>Flesh foods.—</i>				
Egg, white, hen's	0.11	Trace	..
„ yolk, hen's	4.06	2.22	54.7
Fish, muscle	0.72	0.28	38.9
Beef, muscle	2.10	0.73	34.8
Sheep, muscle	3.16	1.98	62.7
<i>Milk and milk products.—</i>				
Milk, cow's	0.24	Trace	..
Skim milk powder	1.43	0.64	44.8
<i>Miscellaneous foodstuffs.—</i>				
Betel leaves	<i>Piper betle</i>	8.33	3.56	42.8
Coco-nut water	0.03	0.01	33.3
Jaggery	11.40	1.71	15.0
' Pappads '	A preparation from black gram (<i>Phaseolus mungo</i>).	21.90	2.11	9.7
Yeast, dried	28.50	12.21	42.8

It will be seen from the above table that the foodstuffs vary widely in their 'available' iron content, and that they do not display any distinct group characteristics. In general, it may be stated that leafy vegetables, usually considered good sources of iron, contain a low percentage of 'available' iron. Yet they furnish, bulk for bulk, as much or even more 'available' iron than most other foodstuffs. Condiments and spices, though rich in iron, resemble leafy vegetables in containing a comparatively low percentage of 'available' iron. In cereals, on the average, one-third of the iron is 'available', a notable exception being ragi (*Eleusine coracana*), in which 'available' iron amounts to only 10 per cent of total

iron. Parboiled rice, beaten rice (i.e., rice which is beaten to produce rice flakes) and puffed rice have a lower percentage of available iron than raw rice; it must, however, be stated that the different rice preparations were not made from the same sample of paddy. In pulses, roots and tubers, other vegetables, and fruits, roughly about 40 per cent of total iron is 'available', while in nuts and oilseeds the average percentage 'availability' is in the neighbourhood of 30.

The results recorded here do not correspond at all closely with those reported by Shackleton and McCance (1936). On the other hand, there is a much closer correspondence between these results and those of Elvehjem and his co-workers, whose method was closely followed in the investigation.

SUMMARY.

(1) One hundred common Indian foodstuffs have been analysed for their 'available' iron by a chemical method involving the use of α, α' -dipyridine. The method finally evolved by Kohler *et al.* has been improved.

(2) The foodstuffs analysed were found to vary widely as regards the percentage of total iron 'available'; in general, leafy vegetables and condiments and spices, usually considered good sources of iron, show low percentage 'availability', while the other groups of foodstuffs contain iron of which about 30 to 40 per cent are 'available'. Percentage 'availability' in the various groups of foodstuffs was very variable.

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PHYTIN-PHOSPHORUS CONTENT OF INDIAN FOODSTUFFS.

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PHYTIN (calcium magnesium salt of inositol phosphoric acid), first isolated by Posternak (1903), is present in various foodstuffs as a reserve material containing phosphorus in organic combination. It was once thought to be an easily assimilable form of phosphorus and has been administered as a 'tonic' (see Plimmer, 1913). Plimmer (*loc. cit.*) found that phytic acid was not hydrolysed by enzymes in the digestive tract of animals. Cereal brans, which are rich in phytin, contain an enzyme, phytase, capable of splitting phytin into inositol and phosphoric acid.

A number of experiments carried out in recent years on the relation of cereals to rickets have suggested the existence of a rachitogenic (anti-calcifying) factor in cereals (Mellanby, 1931). Bruce and Callow (1934) have explained the 'anti-calcifying' effect of cereals on the grounds that the phytin phosphorus of cereals is less available to the animal organism than the inorganic phosphorus. Treatment of oatmeal with 1 per cent HCl destroyed its 'anti-calcifying' effect in proportion to the amount of phytin hydrolysed. Harris and Bunker (1935), however, observed no correlation between the degree of rickets produced in rats by a maize diet and the absolute or relative amounts of phytin phosphorus which the diet contained.

McCance and Widdowson (1935) investigated phosphorus exchanges in three adults and one child and found that 20 to 60 per cent of the phytin were excreted unchanged in the faeces. Lowe and Steenbock (1936a) observed that germinated autolysed maize, immature maize, and HCl-treated maize are less rachitogenic than mature maize, owing to an increased content of inorganic phosphorus. There was a direct relation between the type of maize given and the anti-rachitic effect of the ration, which bore an inverse relation to the phytin content. In a later paper Lowe and Steenbock (1936b) record the fact that phytin phosphorus is not completely unavailable to the rat, which may be due, as Patwardhan (1937) has shown, to the presence of phytase in the intestines.

Phytin thus seems to be a relatively unavailable form of phosphorus. In India ordinary diets are largely composed of cereals and pulses, and phytin phosphorus may form a large percentage of total phosphorus. A diet which appears to be rich in phosphorus as determined by ordinary routine chemical analysis may in reality be deficient in phosphorus in the physiological sense. Hence an investigation of the phytin-phosphorus content of common Indian foodstuffs was undertaken.

METHOD.

The method adopted was essentially that of McCance and Widdowson (*loc. cit.*) and Young (1936) with a few modifications. The method is as follows:—

The phytin phosphorus, which is extracted with dilute HCl, is precipitated as ferric phytate with a known amount of ferric chloride, and the excess determined colorimetrically as thiocyanate. The number of milligrams of iron precipitated is equal to the number of milligrams of phytin phosphorus (Young, *loc. cit.*).

Reagents.—

1. $\frac{N}{2}$ HCl and $\frac{N}{6}$ HCl.
2. NaOH solution 40 per cent.
3. Standard FeCl_3 in NHCl solution.

A solution of A. R. FeCl_3 in NHCl is prepared and the iron determined gravimetrically. By suitable dilution with NHCl a solution containing 1.25 mg. of iron per c.c. is prepared.

4. 20 per cent KCNS.
5. Amyl alcohol.
6. Concentrated iron-free HNO_3 .
7. Standard iron solution. 1 c.c.=0.05 mg. Fe.

Procedure.—Samples for estimation were ground to a fine powder in the case of cereals, pulses, condiments, and spices. Vegetables, which have a high moisture content, were dried in the sun, ground to a fine powder and sieved to pass through a fine mesh. Total phosphorus was estimated volumetrically. Ten grammes of test material were shaken in a 250 c.c. glass-stoppered bottle with 100 c.c. $\frac{N}{2}$ HCl for two hours to extract the phytic acid. It was then transferred to a 100-c.c. centrifuge tube and centrifuged for two hours or more to obtain a clear centrifugate; 25 c.c. of the supernatant were pipetted into a 50 c.c.-measuring-flask and neutralized to phenolphthalein with NaOH, rendered slightly acid with $\frac{N}{6}$ HCl and made up to mark with distilled water.

Twenty c.c. of the above solution were pipetted into a 35 c.c.-test-tube, and 4 c.c. of the standard FeCl_3 -HCl solution added. The tube was then heated in a rack in a boiling water-bath for 15 minutes with the level of the water above that of the contents of the tube. After cooling for 15 minutes in a bath of cold water, the contents of the tube were made up to 50 c.c. in a measuring flask with distilled water and filtered over a dry filter into a dry flask. Iron was estimated in the filtrate by the thiocyanate-amyl-alcohol method. The amount of iron necessary

for precipitation of phytin is calculated therefrom; this represents the phytin phosphorus (Young, *loc. cit.*).

The results are expressed as milligrams per 100 grammes of the edible portion and on a fresh weight basis. The moisture, total phosphorus, phytin phosphorus, and the percentage of the total phosphorus existing as phytin phosphorus, are given in the accompanying Table:—

TABLE.

Moisture, total phosphorus, phytin phosphorus, and the percentage of the total phosphorus existing as phytin phosphorus.

	Name of foodstuff.	Botanical name.	Moisture, per cent.	Total phos- phorus, mg. per cent.	Phytin phos- phorus, mg. per cent.	Phytin, per cent of total phos- phorus.
<i>A. Cereals.—</i>						
1	Bajra or cambu ..	<i>Pennisetum typhoideum</i>	10.37	328.3	246.0	74.92
2	Barley ..	<i>Hordeum vulgare</i>	10.93	212.0	66.0	31.13
3	Cholam ..	<i>Sorghum vulgare</i>	13.05	232.8	206.0	88.49
4	Italian millet ..	<i>Setaria Italica</i>	10.10	320.1	213.4	66.68
5	Maize, tender ..	<i>Zea mays</i>	79.36	104.2	8.6	8.25
6	„ mature ..	„	10.57	363.4	353.0	97.14
7	Oats ..	<i>Avena sativa</i>	11.32	400.4	124.4	31.07
8	Ragi ..	<i>Eleusine coracana</i>	13.53	272.5	246.0	90.26
9	Rice, raw milled ..	<i>Oryza sativa</i>	12.51	111.5	58.0	52.02
10	„ parboiled ..	„	13.26	147.2	85.1	57.81
11	„ black puttu ..	„	11.78	253.0	213.9	84.55
12	„ beaten ..	„	11.46	239.0	187.6	78.48
13	„ puffed ..	„	14.20	169.3	85.6	50.56
14	Wheat, whole ..	<i>Triticum vulgare</i>	13.16	298.3	193.6	64.91
<i>B. Roots.</i>						
15	Arrowroot flour ..	<i>Maranta arundinacea</i>	16.51	17.0	0	0
16	Tapioca flour ..	<i>Manihot utilissima</i>	11.73	66.5	0.5	0.75

TABLE—*contd.*

Name of foodstuff.		Botanical name.	Moisture, per cent.	Total phos- phorus, mg. per cent.	Phytin phos- phorus, mg. per cent.	Phytin, per cent of total phos- phorus.
<i>C. Pulses.—</i>						
17	Bengal gram ..	<i>Cicer arictinum</i>	11.44	280.3	185.7	66.24
18	„ roasted ..	„	11.78	276.8	140.9	50.89
19	Black gram ..	<i>Phaseolus mungo</i>	12.06	401.4	249.7	62.22
20	Cow gram ..	<i>Vigna catiāng</i>	11.73	478.5	258.0	53.92
21	Field bean, white	<i>Dolichos lablab</i>	10.23	417.5	248.2	59.47
22	Green gram ..	<i>Phaseolus radiatus</i>	11.20	293.2	180.1	61.42
23	Horse gram ..	<i>Dolichos biflorus</i>	11.93	249.6	120.9	48.42
24	Lentil ..	<i>Lens esculenta</i>	12.29	284.3	216.0	75.98
25	Peas, roasted ..	<i>Pisum sativum</i>	9.30	351.2	130.5	37.15
26	Red gram ..	<i>Cajanus indicus</i>	11.66	263.6	200.3	75.97
27	Soya bean ..	<i>Glycine hispida</i>	9.23	420.5	213.8	50.86
<i>D. Leafy vegetables.—</i>						
28	Cabbage leaves ..	<i>Brassica oleracea capitata</i>	90.20	48.9	1.1	2.25
29	Coriander leaves ..	<i>Coriandrum sativum</i>	87.90	64.3	0	0
30	Curry leaves ..	<i>Murraya koenigii</i>	66.31	57.3	3.5	6.11
31	Fenugreek leaves	<i>Trigonella foenumgraecum</i>	81.78	52.5	0	0
32	Ipomea leaves ..	<i>Ipomoea reptans</i>	88.20	42.9	0	0
33	Lettuce leaves ..	<i>Lactuca sativa</i>	92.94	27.8	0	0
34	Mint leaves ..	<i>Mentha viridis</i>	82.99	45.8	3.8	8.30
35	Parsley leaves ..	<i>Petroselinum sativum</i>	68.42	150.5	16.6	11.04
36	Sesbania leaves ..	<i>Sesbania grandiflora</i>	76.73	82.6	0	0
37	Spinach leaves ..	<i>Spinacea oleracea</i>	91.66	30.0	0.8	2.67
<i>E. Root vegetables and tubers.—</i>						
38	Beetroot ..	<i>Beta vulgaris</i>	83.81	51.1	0	0
39	Carrot ..	<i>Daucus carota</i>	86.00	35.4	0	0
40	Parsnip ..	<i>Pastinaca sativa</i>	72.41	67.5	4.4	6.52

TABLE—contd.

	Name of foodstuff.	Botanical name.	Moisture, per cent.	Total phos- phorus, mg. per cent.	Phytin phos- phorus, mg. per cent.	Phytin, per cent of total phos- phorus.
	<i>E. Root vegetables and tubers—concl'd.</i>					
41	Potato ..	<i>Solanum tuberosum</i>	74.73	51.1	19.1	37.38
42	Radish, pink ..	<i>Raphanus sativus</i>	90.76	19.2	0	0
43	„ white ..	„	94.41	25.0	0	0
44	Yam, elephant ..	<i>Amorphophallus campanulatus</i> .	78.79	47.5	4.6	9.69
	<i>F. Other vegetables.—</i>					
45	Brinjal ..	<i>Solanum melongena</i>	91.49	42.9	4.7	10.95
46	Celery stalks ..	<i>Apium graveolens rapaceum</i>	93.53	36.9	1.2	3.25
47	Cluster beans ..	<i>Cyamopsis psoralioides</i>	82.45	65.1	3.9	5.99
48	Double beans ..	<i>Faba vulgaris</i>	73.75	149.1	8.1	5.43
49	French beans ..	<i>Phaseolus vulgaris</i>	91.43	27.2	0	0
50	Ipomea stalks ..	<i>Ipomoea reptans</i>	93.67	25.2	0	0
51	Jack-fruit seeds ..	<i>Artocarpus integrifolia</i>	51.60	85.9	40.4	47.03
52	Lady's fingers ..	<i>Hibiscus esculentus</i>	87.95	74.2	0	0
53	Raw plantain ..	<i>Musa paradisiaca</i>	83.24	26.8	0	0
54	Spinach stalks ..	<i>Spinacia oleracea</i>	93.37	15.2	0	0
	<i>G. Condiments and spices.—</i>					
55	' Arisithippili ' ..	<i>Piper clusii</i>	12.53	369.2	56.2	15.23
56	Chillies, green ..	<i>Capsicum annuum</i>	82.60	79.3	3.4	4.31
57	„ dry ..	„	11.31	342.6	71.2	20.78
58	Coriander seeds ..	<i>Coriandrum sativum</i>	8.45	416.1	320.5	77.02
59	Cumin ..	<i>Cuminum cyminum</i>	10.32	460.5	153.3	33.29
60	Fenugreek seeds ..	<i>Trigonella foenumgraecum</i>	11.04	384.9	150.9	39.20
51	Nutmeg ..	<i>Myristica fragrans</i>	14.32	264.3	162.1	61.34
62	Omum (Bishop's weed).	<i>Carum copticum</i>	10.71	309.0	296.7	96.01

TABLE—concl'd.

	Name of foodstuff.	Botanical name.	Moisture, per cent.	Total phos- phorus, mg. per cent.	Phytin phos- phorus, mg. per cent.	Phytin, per cent of total phos- phorus.
	<i>G. Condiments and spices—concl'd.</i>					
63	Pepper, dry ..	<i>Piper nigrum</i>	12.86	197.0	115.1	58.42
64	Turmeric, dry ..	<i>Curcuma longa</i>	13.06	284.0	97.3	34.26
	<i>H. Miscellaneous.—</i>					
65	Bread, white	41.85	56.1	0	0
66	Sago ..	<i>Metroxylon sago</i>	12.20	6.0	0	0
67	Vermicelli	11.21	81.2	17.0	20.93

In some cases, notably those of horse gram, soya bean, mint leaves, spinach leaves, parsnip, potato, elephant yam, brinjal, jack-fruit seeds, and 'Arisithippili', total phosphorus content shows marked variations from figures obtained in a previous investigation (Ranganathan *et al.*, 1937). A higher degree of natural variation in mineral content is to be expected in vegetables than in cereals.

The Table shows that a large proportion of the phosphorus in cereals and pulses exists as phytin, the largest percentage being in mature maize.

The vegetables on the whole contain negligible amounts of phosphorus in this form, the quantity present rarely exceeding 10 per cent, though two notable exceptions are seen in potato and jack-fruit seeds with 37.38 per cent and 47.03 per cent phosphorus as phytin.

The condiments and spices fall in between cereals and pulses and the vegetables, though here again marked exceptions are noticeable, in omum (Bishop's weed) with 96.01 per cent and green chillies with 4.31 per cent as phytin.

SUMMARY.

The phytin-phosphorus content of 67 foodstuffs has been determined. In cereals a high percentage of total phosphorus is present as phytin, while in vegetables, with certain exceptions, the percentage is small or phytin is absent. The 'condiments and spices' investigated in general occupy an intermediate position.

A. R. Sundararajan.

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THE EFFECT ON RATS OF SUPPLEMENTING A SOUTH INDIAN DIET WITH CALCIUM AND PHOSPHORUS.

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TYPICAL poor South Indian diets, largely based on rice, are deficient in mineral salts and particularly in calcium. In a recent paper Aykroyd and Krishnan (1937) showed that the inclusion of calcium lactate greatly enhanced growth and improved the general condition of rats fed on a poor South Indian diet. They also noticed great improvement in the growth of the animals when the basal diet was supplemented by skimmed milk powder and expressed the opinion that 'the value of milk is due in large measure to its high calcium content'.

Sherman (1933) has observed that 'the most practical means of ensuring an abundance of calcium in the dietary is to use milk freely as food and this ensures a good intake of phosphorus and a favourable ratio between calcium and phosphorus at the same time'. Other workers, e.g., Brown and Sohl (1930), hold that calcium and phosphorus must be present in diet in sufficient amounts and in correct relationship to each other to ensure their retention in proper amounts and in correct relations for normal bone deposition.

The present investigation was undertaken in order to study the effect of supplementing the poor South Indian diet with calcium and phosphorus respectively given singly and with both given in combination.

EXPERIMENTAL.

Four groups of 12 young rats, about 50 grammes to 60 grammes in weight, consisting of equal numbers of males and females, were given the following diet,

corresponding to that used by Aykroyd and Krishnan (*loc. cit.*) in their experiments:—

	Oz.
Raw milled rice	21.00
Dhal arhar (<i>Cajanus indicus</i>)	0.70
Black gram (<i>Phaseolus mungo</i>)	0.70
Brinjal (<i>Solanum melongena</i>)	1.00
Amaranth leaves (<i>Amaranthus gangeticus</i>)	0.50
Raw plantains (<i>Musa paradisiaca</i>)	0.50
Gingelly oil	0.10
Coco-nut (<i>Cocos nucifera</i>)	0.05
Meat (mutton)	0.06

This diet, which corresponds roughly to the daily diet of an adult of the poorer classes in South India, was mixed and fed in the proportions indicated.

Group I was given the basal diet alone. Group II was given the basal diet supplemented by 7.0 grammes of calcium lactate, i.e., the food offered to each individual rat daily contained about 0.15 gramme of calcium lactate supplement. Group III was given the basal diet fortified by 3.2 grammes of alkaline potassium phosphate, representing an addition of about 0.07 gramme to the daily food allowance of each rat. Group IV was given the basal diet containing both supplements in similar quantities.

The animals were exposed to the sun occasionally. The experiment lasted for ten weeks during which weights were recorded weekly. Table I and the accompanying figures show the average rate of increase in weight in the four groups during the experimental period.

During the first four weeks group II showed the greatest gains in weight, closely followed by the fourth group. During the remaining six weeks group IV showed the greatest increase. The difference between groups II and IV was, however, not marked.

Batches of three animals (males) from each group were kept in metabolism cages [Coonoor type, described by Niyogi and co-workers (1932)] for four weeks from the second to the fifth week of the experiment. Total food intake was calculated by deducting the residue left each morning from the quantity given on the previous day (dry weights). The same investigation was repeated for batches of three females from each group for four weeks from the sixth to the ninth week. It was noticed throughout that the animals from groups II and IV consumed more food than those from groups I and III, group III consuming the least.

The rejected food, fæces, and urine of each group were stored in separate vessels for seven days. when chemical analysis of calcium and phosphorus in the food and excreta was carried out, and their retention calculated. At the end of ten weeks the animals were bled to death under ether anæsthesia, blood from the heart being taken for the estimation of serum calcium and phosphorus. The thyroid with the parathyroids and pieces of the small intestine were removed for histopathological examination.

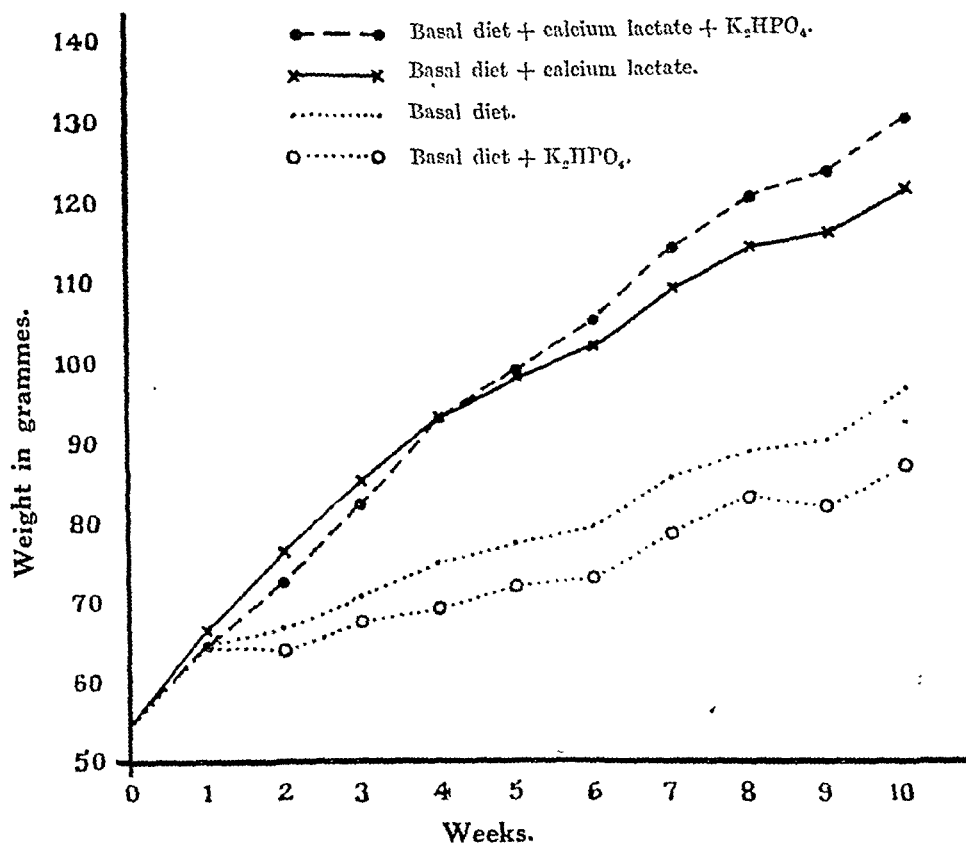


Chart showing the average rate of increase in weight of the four groups of experimental animals for ten weeks.

TABLE I.
Increase in weight in ten weeks.

Group.	Initial weight, g.	1st week, g.	2nd week, g.	3rd week, g.	4th week, g.	5th week, g.	6th week, g.	7th week, g.	8th week, g.	9th week, g.	10th week, g.
I ..	54.6	64.4	66.5	70.4	74.7	77.1	79.1	85.3	88.7	90.0	96.5
II ..	54.6	66.8	76.3	85.0	92.7	98.0	101.8	109.0	114.1	115.8	121.1
III ..	54.6	64.1	63.7	67.5	69.1	71.8	72.8	78.5	83.2	82.0	87.1
IV ..	54.6	64.3	72.4	82.0	92.4	98.7	104.8	113.9	120.3	123.3	129.7

The estimation of calcium in the food and faeces was carried out by the standard methods of ashing and precipitation as calcium oxalate and titration with N-10 potassium permanganate solution; that of phosphorus by precipitation as phosphomolybdate, dissolving in N-10 alkali and titration with N-10 acid, while that of total phosphorus and calcium in urine was done by the combined method of Greenwald and Gross (1925). The serum calcium was determined by Kramer and Tisdall's method (1921), and the blood phosphorus was estimated by the Bell-Doisy method modified by Briggs (1922).

The results of the analysis are set out in Table II :—

TABLE II.

Food intake and calcium (CaO) and phosphorus (P₂O₅) intake, excretion and retention during four weeks (males and females).

Group.		Food intake, g.	Total calcium in food, g.	Total calcium excreted, g.	Total calcium retained, g.	Total phosphorus in food, g.	Total phosphorus excreted, g.	Total phosphorus retained, g.
MALES :—								
1st week	I	215	0.199	0.024	0.175	0.595	0.265	0.330
	II	220	0.567	0.050	0.517	0.620	0.146	0.474
	III	210	0.165	0.016	0.149	0.958	0.396	0.562
	IV	220	0.665	0.097	0.568	1.045	0.392	0.653
2nd week	I	183	0.174	0.019	0.155	0.498	0.183	0.315
	II	208	0.536	0.061	0.475	0.566	0.186	0.380
	III	141	0.103	0.018	0.085	0.666	0.292	0.374
	IV	226	0.734	0.103	0.631	1.067	0.433	0.634
3rd week	I	213	0.187	0.040	0.147	0.575	0.180	0.395
	II	247.5	0.653	0.113	0.540	0.673	0.159	0.514
	III	191.5	0.161	0.053	0.108	0.886	0.397	0.489
	IV	278	0.778	0.142	0.636	1.280	0.416	0.864
4th week	I	206	0.208	0.022	0.186	0.587	0.325	0.262
	II	241	0.911	0.234	0.677	0.699	0.250	0.449
	III	189	0.104	0.026	0.078	0.876	0.512	0.364
	IV	233	0.705	0.234	0.471	1.071	0.645	0.426

TABLE II—*concl'd.*

Group.		Food intake, g.	Total calcium in food, g.	Total calcium excreted, g.	Total calcium retained, g.	Total phosphorus in food, g.	Total phosphorus excreted, g.	Total phosphorus retained, g.
FEMALES :—								
1st week	I	196	0.202	0.023	0.179	0.543	0.393	0.150
	II	238	0.699	0.151	0.548	0.659	0.228	0.431
	III	202	0.242	0.026	0.216	0.929	0.554	0.375
	IV	227	0.629	0.163	0.466	1.044	0.676	0.368
2nd week	I	185	0.171	0.012	0.159	0.496	0.255	0.241
	II	234	0.727	0.182	0.545	0.628	0.219	0.409
	III	171	0.172	0.013	0.159	0.805	0.422	0.383
	IV	225	0.680	0.153	0.527	1.060	0.522	0.538
3rd week	I	240	0.222	0.061	0.161	0.638	0.388	0.250
	II	263	0.817	0.249	0.568	0.699	0.437	0.262
	III	239	0.201	0.033	0.168	1.153	0.618	0.535
	IV	250	0.840	0.254	0.586	1.207	0.836	0.371
4th week	I	248	0.250	0.028	0.222	0.745	0.407	0.338
	II	281	0.944	0.358	0.586	0.844	0.530	0.314
	III	229	0.269	0.042	0.227	1.146	0.636	0.510
	IV	245	0.884	0.370	0.514	1.226	0.714	0.512

From Table II it will be seen that retention of calcium was greatest for males in group IV (except in the 4th week). Group II was next in order, then group I and lastly group III. The order for phosphorus retention was as follows: Groups IV, III, II, and I. As regards females, group II showed the greatest calcium retention, closely followed by group IV, while in groups I and III retention was less, being almost equal in these groups (except in the 1st week). The maximum amounts of phosphorus were retained by groups III and IV and the minimum by group I. Group II (female) showed a higher degree of retention in the 1st and 2nd weeks than in the later period.

According to Karelitz and Sohl (1927), the ratio $\frac{\text{calcium retained}}{\text{phosphorus retained}}$ is of greater significance than the retention of calcium or phosphorus considered individually,

In Table III the ratio Ca : P retained has been calculated from the CaO and P₂O₅ figures in Table II :—

TABLE III.

Ca : P retained in the various groups.

Group.		2nd week.	3rd week.	4th week.	5th week.
Males	I	0·86	0·80	0·60	1·15
	II	1·29	2·02	0·71	2·30
	III	0·42	0·37	0·36	0·35
	IV	1·42	1·62	1·20	1·71
		6th week.	7th week.	8th week.	9th week.
Females	I	1·81	1·07	1·05	0·98
	II	2·08	2·03	3·53	3·05
	III	0·94	0·67	0·51	0·72
	IV	2·12	1·63	2·67	1·64

Estimations of calcium and phosphorus in the blood are given in Table IV. There was a striking difference between the blood calcium levels in the groups receiving calcium lactate (II and IV) and those in the other two groups.

TABLE IV.

Calcium and phosphorus per 100 c.c. of blood.

Group.		Calcium (average), (mg. per cent).	Phosphorus (average), (mg. per cent).
I	..	6·12	12·89
II	..	13·16	10·39
III	..	8·32	11·67
IV	..	14·00	9·33

Histological study of sections of the parathyroid and thyroid glands revealed the following :—

PARATHYROIDS.

Group I.—The glands appeared to be enlarged. The principal cells were loosely and irregularly arranged, very few oxyphil cells being present,

PLATE XXII.



Fig. 1.—Parathyroid gland. Group I. Showing fibrosis and hypo-active condition of the gland.

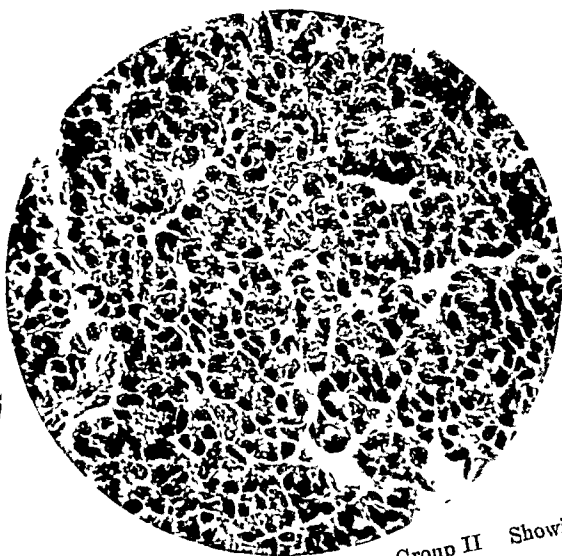


Fig. 2 —Parathyroid gland. Group II Showing normal structure.

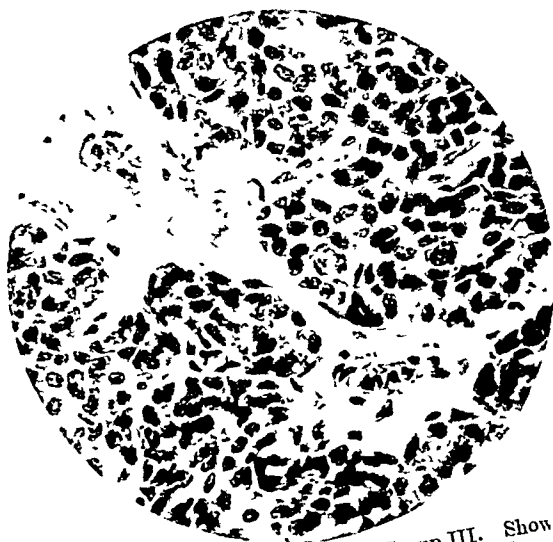


Fig. 3.—Parathyroid gland. Group III. Showing fibrosis and hypo-active condition of the gland.

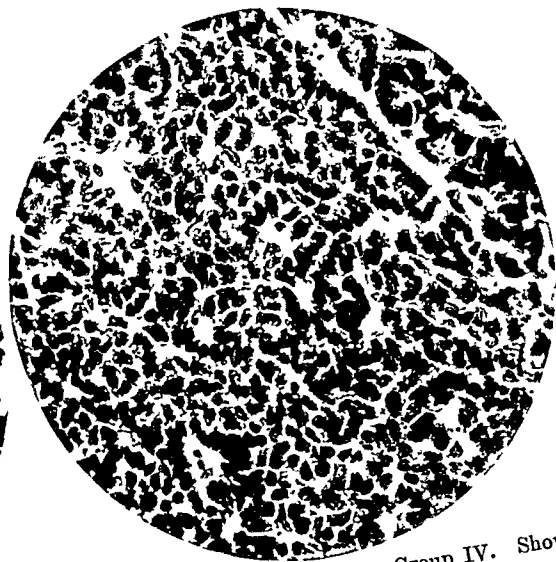


Fig. 4 —Parathyroid gland. Group IV. Showing normal structure.

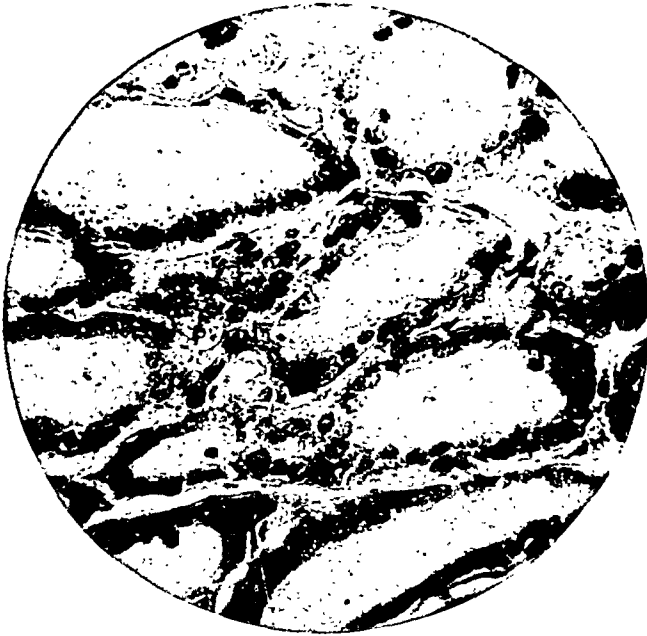


Fig. 5.—Thyroid gland. Group I. Showing colloid storage and hypo-active condition of the gland.

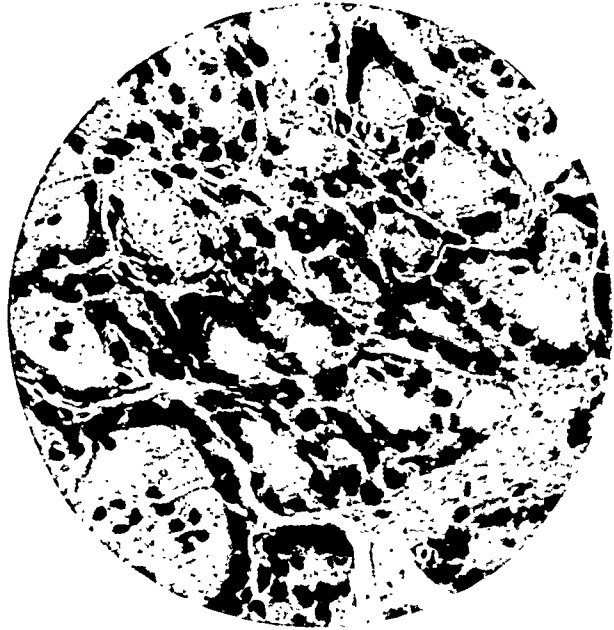


Fig. 6.—Thyroid gland. Group II. Showing normal structure.

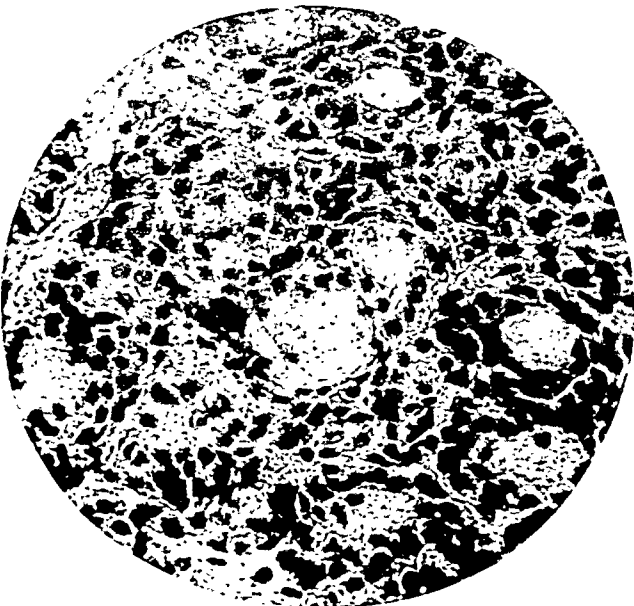


Fig. 7.—Thyroid gland. Group III. Showing absence of colloid material and inert condition of the gland.



Fig. 8.—Thyroid gland. Group IV. Showing normal structure.



Fig. 9.—Small intestine. Group I. Showing thin musculature and some distortion of the villi.



Fig. 10.—Small intestine. Group II. Showing thick musculature and normal villi.



Fig. 11.—Small intestine. Group III. Showing very thin musculature and distortion of the villi.



Fig. 12.—Small intestine. Group IV. Showing very thick musculature and some distortion of the epithelium of the villi.

The blood sinuses were very few in number, many having apparently been replaced by strands of fibrous tissue.

Group II.—The glands were of normal size. The principal cells were compact, and there was a tendency towards acinar formation. The oxyphil cells could be seen here and there. The blood sinuses were numerous, and fibrous bands were very few and slender.

Group III.—The glands were enlarged. The principal cells were not compactly arranged, being separated by a prominent reticular network. A few oxyphil cells were visible. The blood sinuses were scanty in number, and there were many large fibrous tissue bundles dividing the glands into lobular portions.

Group IV.—The glands were normal in size. The principal cells were compact with a tendency towards acinar formation. Oxyphil cells were observed here and there. The blood sinuses were quite prominent, though less in number than in group II. The fibrous tissue bundles were few and narrower than in groups I and III.

Van Gieson's method of staining demonstrated the presence of fibrous tissue clearly and vividly in contrast with the glandular and elastic tissue in the glands.

THYROIDS AND INTESTINES.

Histological examination of the thyroids revealed appearances in groups I and III suggestive of hypofunction; these were more marked in group III. In groups II and IV the glands appeared normal. In groups I and III the small intestine showed a tendency towards thinness of the muscular coat, with prominence and distortion of the villi; lymphoid cells were few in number; there were no distinct goblet cells, and the granules in the cells of Paneth were indistinct. No abnormalities of this nature were visible in the other two groups. The changes in groups I and III are suggestive of inanition.

DISCUSSION.

The reported results show that a diet largely based on rice, typical of the diets consumed by the poorer classes in many parts of India, is deficient in calcium and to a less serious extent in phosphorus. Aykroyd and Krishnan (*loc. cit.*) observed the 'growth-enhancing' effect which followed the addition of calcium lactate to the 'poor Madrassi diet', and considered that the improvement brought about in Indian school children by the provision of skimmed milk was due in large measure to the calcium supplied by the milk. We suggest that the phosphorus present in milk also played a part in producing this effect.

In the present experiment the addition of alkaline phosphate alone had no supplementary effect on growth, and indeed growth on the basal diet supplemented by phosphate was poorer than growth on the basal diet alone. Mackay and Oliver (1935) observed that excess of inorganic phosphate (90 mg. per 100 g. of food) caused damage to the kidneys of rats. Salvesen *et al.* (1924) found that, if alkaline phosphates are used, no changes in the calcium and inorganic phosphorus content of the blood follow as a result of giving small doses over a long period. We used alkaline potassium phosphate, which is the least toxic of the phosphate salts, the

dose being about 80 mg. per 100 g. of food. The poor performance of group III cannot therefore be explained as a result of toxic action. Karelitz and Sohl (*loc. cit.*) noticed that rats consumed less food when phosphate was added to their diet. In our experiments, the animals in group II consumed the smallest quantity of food and the histological changes in the intestines and the thyroid gland were suggestive of inanition.

The possibility of supplementing a diet deficient in calcium by the addition of pure calcium salts has been the subject of controversy. Sherman (1920*a* and *b*) and Steenbock *et al.* (1923) considered that deficiencies of calcium and phosphate can be made good by supplying pure mineral salts. The latter workers observed that while there is no difference in the availability for rats of calcium salts such as carbonate, phosphate, silicate or sulphate when these are fed in liberal amounts, calcium lactate 'did not constitute an especially good source of calcium for the animal'. In our experiments calcium lactate proved an effective supplement.

The relatively good growth shown by group II, receiving an addition of calcium lactate alone, may be considered in the light of the observation of Sherman and Quinn (1926) that 'calcium intake is a limiting factor as regards storage of both calcium and phosphorus in the body, which is increased when calcium only is added to the food'. In our experiments group IV, receiving both calcium and phosphate, showed slightly better growth than the group receiving calcium alone. Kon (1937) has remarked that 'the metabolism of calcium and phosphorus are closely interrelated, and it is quite understandable that . . . the availability of the calcium of milk may be profoundly influenced and even reversed by marked variations in the quantity and quality of the phosphorus component of the diet'. Hence we would emphasize the importance of considering phosphorus requirements in conjunction with requirements of calcium.

There was a relation between calcium retention and the growth rate in our experiments. The ratio $\frac{\text{calcium retained}}{\text{phosphorus retained}}$ was, however, felt to be of more importance in relation to growth, than the retention of calcium or phosphorus alone. The retention of these elements may be increased after a period of depletion, or storage may occur for the formation of deposits subject to call as the needs of the body dictate. Accordingly, attention was given to the $\frac{\text{Ca}}{\text{P}}$ (retained) ratio, which was found in these experiments to be related to the growth rate.

According to Karelitz and Sohl (*loc. cit.*), the normal $\frac{\text{Ca}}{\text{P}}$ (retained) ratio is about 1.7. Sohl and Bennett (1927) give the higher figure of 2.85 as normal. If the normal range of the ratio is taken as 1.7 : 2.85, the ratios in group II (males) and group IV (females) are found to be normal, or very nearly so, throughout the experiments. Group IV (males) showed normal ratios alternating with lower ones, i.e., they showed a relatively higher retention of phosphorus. Group II (females) displayed for the first two weeks of the experiment normal ratios; during the following two weeks ratios were higher, indicating higher retention of calcium. In groups I and III the ratios were consistently low, indicating poor calcium retention. As previously observed, there was a relation between the range of the ratios and the rate of increase in weight.

In groups I and III serum calcium was very low (6.12 mg. and 8.32 mg. per cent respectively). In groups II and IV it was rather high (13.16 mg. and 14.00 mg. per cent respectively). The phosphorus content of the serum was in inverse ratio to calcium content, being highest for group I (12.89 mg. per cent), next highest for group III (11.67 mg. per cent), and 10.39 mg. and 9.33 mg. for groups II and IV respectively. Karelitz and Sohl (*loc. cit.*) observed a fall of serum calcium to 5.5 mg. to 7.0 mg. per cent, and rise of serum phosphorus to 14.5 mg. to 16.0 mg. per cent, three days after the administration of phosphate to the rats. Salvesan and his co-workers (*loc. cit.*) found that large doses of phosphate (0.40 g. to 0.70 g. of phosphorus per kilo of body-weight), administered for a period of one to two days, produced symptoms of tetany, the serum calcium increasing by 2.5 mg. to 3.9 mg. per cent, and the serum calcium decreasing by 1.51 per cent to 1.81 per cent. They also noted, however, that small doses of phosphate given over a long period produced no clinical symptoms, while no changes in the calcium and inorganic phosphate content of the blood occurred if alkaline phosphates were given. In our experiments groups III and IV received alkaline potassium phosphate, and while group III showed a low calcium and a high phosphorus figure, group IV showed the reverse. In group I, which received no phosphate, serum phosphorus was higher, and serum calcium lower, than in group III which was given phosphate. It is thus apparent that the phosphorus intake cannot be held responsible for the serum calcium and serum phosphorus levels observed in the experimental animals.

There has been much controversy whether serum calcium can be increased by addition of calcium salts to the diet. While Denis and Corley (1925) and Halverson and co-workers (1917) maintained that no increase of calcium content of blood serum in animals was possible by supplementing food with calcium or calcium-rich food, Hjost (1925) and Kahn and Roe (1926) demonstrated without doubt that calcium salts (lactate) administered orally to rats produced a definite elevation of the level of blood calcium. This observation has found ample corroboration in our experiments, since groups II and IV, which received supplements of calcium lactate, showed much increased levels of blood calcium.

The fall in the serum calcium and the corresponding rise in serum phosphorus in groups I and III can perhaps be explained in terms of parathyroid insufficiency, the characteristic features of which, according to Salvesan (1923), are a low calcium and high phosphorus level in the blood. Maximow and Bloom (1934) also think that the increased serum phosphorus is as important as the fall in calcium in tetany (or parathyroid deficiency). The structural changes in the parathyroid glands of groups I and III, which were suggestive of hypofunction, also corroborate the above theory. But Karelitz and Sohl (*loc. cit.*) noticed that a high inorganic phosphorus and low calcium blood serum content was associated with a spastic condition of the limbs in rats, and Hastings and Murray (1921) found that when serum calcium was below 7 mg. per 100 c.c. tetany occurred. But in spite of conditions favourable for its development, tetany did not occur in groups I and III. Rats appear to be a species in which tetany can be produced experimentally with great difficulty.

The parathyroid glands of the other two groups of animals showed normal histological appearances. It is possible that the enlargement of the parathyroids,

the inert condition of the thyroid gland shown by the absence of colloid material, and the thin muscular coat of the small intestine, which were present in group III and to a lesser extent in group I, were due to inanition resulting from malnutrition. This is in accordance with the findings of Jackson (1916) and McCarrison (1928).

SUMMARY.

(1) Addition of calcium enhances the nutritive value of Indian diets based on rice, as assessed by the growth of groups of young rats. This effect is slightly increased if phosphorus is also added at the same time. The addition of phosphorus alone has no supplementary effect on growth.

(2) In the groups receiving the basal diet, and the basal diet supplemented by phosphorus, changes indicative of hypofunction were observed in the parathyroid glands. These changes were not observed when the diet was supplemented by calcium, or by calcium and phosphorus given together.

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STUDIES ON BLOOD CHOLINE ESTERASE.

BY

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ENGELHARDT and LOEWI (1930) and MATTHES (1930) confirmed the previous observation of Dale (1914) that the destruction of acetylcholine (A.C.) in the blood was probably due to its hydrolysis into choline and acetic acid by an esterase in the blood. Stedman *et al.* (1932) isolated the enzyme in a purer state and demonstrated the specificity of the enzyme as it hydrolysed only choline esters. They showed that unlike other esterases, such as pancreatic lipase, the enzyme had no action on esters like methyl butyrate and they proposed to name it 'choline esterase' (C.E.). Since this observation of Stedman and his co-workers considerable work has been done to determine the C.E. activity of bloods obtained from different sources. The work that has been done so far has recently been very well reviewed by McGeorge (1937). The specific property of C.E. of destroying A.C. indicates that it probably plays an important rôle in mammalian physiology and pathology. It was, therefore, decided to investigate the C.E. of blood under different physiological conditions.

METHODS.

There are at present three important methods by which the C.E. activity of tissues can be determined, viz., (I) the continuous titration method, (II) the gasometric method, and (III) the pharmacological method.

The work was originally started with the aim of studying the C.E. activity of the serum and the continuous titration method (Stedman *et al.*, 1933) was followed. As the method was not suitable for estimation of C.E. activity of highly pigmented solutions like blood or R.B.Cs., the study of which was later thought necessary, the gasometric and pharmacological methods were also resorted to.

I. *Continuous titration method* (Stedman *et al.*, 1933).—In a glass water-bath maintained at 30°C. was placed a conical flask (250 c.c.) containing CO₂-free water (100 c.c.) to which were added 5 drops of aqueous solution of cresol red (Clark, 1925) and 25 per cent aqueous solution of A.C. bromide (1 c.c.). The pH of the mixture was brought to 8.0 by the addition of requisite amounts of 0.02-N caustic

soda. A standard borate buffer was kept under similar conditions for comparison. Serum under examination (1 c.c.) was next added to the mixture and the pH readjusted to 8.0 by the addition of standard alkali. The hydrolysis of the substrate proceeded immediately with the liberation of acetic acid; the standard alkali was added drop by drop from the burette to maintain the pH of the mixture at the original level and the amount of the standard alkali used in 20 minutes was noted. If the serum was pigmented 1 c.c. of it was added to the standard borate buffer also which was kept for comparison.

II. *Gasometric method* (Stedman and Stedman, 1935).—Bicarbonate Ringer's solution (5 c.c.) and 1 c.c. of the liquid under investigation were added to each flask of the Barcroft's differential manometer. Bicarbonate Ringer's solution (0.5 c.c.) and 1 per cent aqueous solution of A.C. bromide (0.5 c.c.) were then added to the side tube of one of the flasks and the second flask was also treated similarly except that water was used in place of aqueous solution of A.C. bromide. The apparatus was then assembled and immersed in a water-bath at 30°C. Both the flasks were so arranged that they could be evacuated and filled repeatedly with a gas mixture composed of 95 per cent N and 5 per cent CO₂ till most of the air from the apparatus had been replaced by the gas mixture. The stop-cocks of the flasks were readjusted, shaking commenced and continued till the equilibrium was reached; the contents of the two compartments of the flasks were then mixed and shaking started again. The amount of CO₂ thus liberated in 30 minutes was measured and reduced to N.T.P. in the usual way.

III. *Pharmacological method*.—For the pharmacological method the *rectus abdominis* muscle of *Rana tigrina* was used. 0.5 c.c. of defibrinated blood diluted to 1:10 was allowed to react at room temperature (27°C.) with an equal volume of 1:10⁴ solution of A.C. for 180 seconds. The mixture was then immediately added to the bath containing the *rectus abdominis* muscle previously sensitized by soaking in 1:3×10⁵ solution of eserine in Ringer's solution. The contraction of the muscle was recorded on a moving drum for exactly 75 seconds after which the drum was stopped and the bath replaced with fresh Ringer's solution. The muscle was allowed to relax and if necessary was pulled lightly to bring it to its normal level. The Ringer's solution was then replaced by eserinated Ringer's solution and the muscle was ready for another application of A.C. The time for which the muscle was kept in eserinated Ringer was 5 minutes. Control applications of A.C. were frequently made to see that the muscle was responding to the same concentration of the drug with the same degree of contraction. The capacity of the bath was 100 c.c.

C.E. CONCENTRATION OF SERA IN DIFFERENT SPECIES AND INDIVIDUALS OF THE SAME SPECIES.

Stedman and Stedman (*loc. cit.*) found that C.E. concentration of sera or blood of different animal species and also amongst individuals of the same species varies considerably though it remains constant for particular individuals for a considerably long period. Verebely (1936) studied about 100 human sera and confirmed the earlier observations of Stedman and his associates. In the present investigations cats', dogs', and guinea-pigs' sera and guinea-pigs' blood have been examined for their C.E. activity by methods I and II respectively. The results obtained are

shown in Tables I and II. It will be seen from the tables that the C.E. activity of sera or of blood of different animal species or among individuals of the same species varies, but remains almost constant for each particular individual for a fairly long period. The results, therefore, confirm the observations made by previous workers.

TABLE I.

Showing the choline esterase activity of cats', dogs', and guinea-pigs' sera in c.c. of 0.02 N NaOH required to neutralize the acid liberated in 20 minutes.

Case number.	Cats' sera.	Dogs' sera.	Guinea-pigs' sera.
1	1.8	3.45	4.5
2	1.5	3.10; 3.2 (10 days after)	4.4
3	1.0	3.00	4.2
4	1.0	2.9; 2.9 (4 " ")	3.5
5	1.0	2.7; 2.8 (12 " ")	3.3; 3.1 (13 days after).
6	..	2.3; 2.1 (15 " ")	2.6; 2.7 (13 " ").
7	..	2.2; 2.2 (24 " ")	2.25
8	..	2.2	2.15; 2.3 (1 month after).
9	..	1.05	2.1
10	2.05

TABLE II.

Showing the C.E. activity of 1 : 5 dilution of guinea-pigs' blood in c.c. of CO₂ evolved during 30 minutes, calculated at N.T.P.

Case number.	CO ₂ .	CO ₂ after 3 days.	CO ₂ after 8 days.	CO ₂ after 29 days.
1	0.1721
2	0.1549	..	0.1516	0.1481
3	0.1516
4	0.1481	0.1429
5	0.1377	0.1380	0.1336	..
6	0.1377	..	0.1410	..
7	0.1377
8	0.1360
9	0.1343
10	0.1326
11	0.1308	..	0.1343	..
12	0.1304
13	0.1291	0.1291	0.1251	..
14	0.1244	0.1244	0.1307	0.1306
15	0.1215	0.1219
16	0.1205	..	0.1240	0.1205
17	0.1119	0.1136	0.1107	0.1117
18	0.1033	0.1033	0.1108	0.1033
19	0.09797	0.09642	..	0.09660

In the above experiments cats' and dogs' blood was taken from a vein, while the guinea-pigs' blood was withdrawn from the heart.

In order to see if there was any difference in the C.E. activity of the arterial and venous blood dogs were anæsthetized with ether and their blood taken out from the femoral vein and the carotid artery. The sera of these were removed carefully with a pipette and the C.E. activity of each estimated by method I. The observations were extended further and changes in the C.E. activity of blood serum after death were also studied in a few experiments. The animals were killed by injecting air intravenously and samples of blood taken from the heart at 20 minutes' and 40 minutes' intervals after death. The results are summarized in Table III :—

TABLE III.

Showing the C.E. activity of dogs' serum in c.c. of 0.02 N NaOH required to neutralize the acid liberated in 20 minutes.

Case number.	Serum from arterial blood.	Serum from venous blood.	Serum from blood taken 20 minutes after death.	Serum from blood taken 40 minutes after death.
1	2.0	2.2	2.2	2.1
2	3.2	3.4	3.0	3.1
3	2.1	2.3
4	2.9	2.8

It will be seen from the above table that the C.E. activity of serum obtained from arterial blood is the same as that of serum obtained from the venous blood and that blood taken from the animal a few minutes after its death retains its C.E. activity.

Stability of the esterase.—Stedman and Stedman (*loc. cit.*) have shown that dilution of blood with water lowers its C.E. activity as the stability of the enzyme is poor in dilute solutions. This observation was confirmed in my experiments. As it is sometimes inconvenient to examine the serum or blood for its C.E. activity immediately after its removal, experiments were designed to see the stability of the enzyme under different conditions of storage.

Samples of dogs' blood serum were therefore stored in a refrigerator for 48 hours and the C.E. activity of the samples determined before and after their storage. It was observed that such storage did not in any way affect the enzyme activity.

In one experiment defibrinated human blood was stored in a refrigerator for 72 hours and its C.E. activity was determined before and after its storage by method II. The blood sample before its storage liberated 0.18666 c.c. of CO_2 calculated at N.T.P. and 72 hours after its storage in the refrigerator it again liberated 0.18666 c.c. of CO_2 calculated at N.T.P., as before.

These results show that the tissues can be stored in a refrigerator for a couple of days without any appreciable loss in their C.E. activity. The esterase was, however, found to be unstable at room temperatures. The activity of the enzyme was found to deteriorate considerably by keeping it at room temperature (27°C . to 30°C .) for 24 hours. Higher temperatures such as 50°C . to 60°C . were found to destroy the enzyme considerably in 15 to 20 minutes.

EFFECT OF DIFFERENCE IN PHYSIOLOGICAL CONDITIONS.

(a) *Effect of sleeplessness.*—Dikshit (1934) has shown that injections of A.C. (0.1 γ to 0.5 γ) into the lateral ventricle of the brain or deeper into the hypothalamic region of cats produced a condition closely resembling sleep; the study of the C.E. activity of bloods of the animals kept awake for a considerably long period was, therefore, thought to be of interest. To carry out the experiment two guinea-pigs were kept in a continuously revolving cage and the C.E. activity of their blood determined before and after the experiment by method II. (In all experiments with guinea-pigs' blood, the blood was obtained by heart puncture and diluted to 1 : 5 with distilled water.) Table IV shows the results:—

TABLE IV.

Showing the effect of sleeplessness on the choline esterase activity of guinea-pigs' blood.

Case number.	CO_2 normal.	CO_2 .
1	0.1119	0.1102 (96 hours after sleeplessness).
2	0.1326	0.1377 (144 „ „ „).

Thus it was found that sleeplessness had no effect on the C.E. activity of blood of the animals.

(b) *Effect of temperature.*—To study the effect of temperature a guinea-pig was kept in the water-bath at 45°C . for 90 minutes and its blood examined for the C.E. before and immediately after the experiment and also 24 hours after the

experiment. The rectal temperature of the animal rose considerably but such a change in the temperature did not in any way modify the activity of the enzyme.

(c) *Effect of starvation and feeding of glucose.*—In a recent publication Quastel *et al.* (1936) have shown that increased glucose concentration increased the choline ester formation activity of tissues *in vitro* and Hebb (1935) observed that concentration of blood sugar affects the amount of enzyme secreted by the pancreas. It was, therefore, thought that glucose feeding in animals, which would raise their blood-sugar level, might bring about some change in the C.E. activity of blood of the animals.

To study the effect of starvation on the C.E. activity of the blood of the animals, three guinea-pigs were starved and the C.E. activity of their blood determined before and after the experiment by method II. Two of these guinea-pigs died after 120 hours' starvation. In the only surviving case (case 2) 6 g. of glucose in water, in two equal portions with an interval of three hours between each dose, were administered, the blood sample being taken after another 3 hours and examined for the C.E. activity.

TABLE V.

Showing the effect of starvation and glucose feeding on the choline esterase activity of guinea-pigs' blood.

Case number.	CO ₂ before fast.	CO ₂ after 50 hours' fast.	CO ₂ after 100 hours' fast.	CO ₂ 3 hours after glucose administration.
1	0.12990	0.12570	0.1170	..
2	0.12050	0.11880	0.09986	0.11360
3	0.14460	0.14120	0.11020	..

From Table V it would appear that there is a slight lowering in the C.E. activity of blood of the animals due to starvation, but side by side determinations by method III showed no such change. Glucose feeding in the only surviving case after 120 hours of starvation shows no significant change in the C.E. content of its blood.

Action of drugs on the C.E. activity of guinea-pigs' blood.

It has been observed by several workers (McGeorge, *loc. cit.*; Matthes, *loc. cit.*) that eserine inhibits the enzyme and this is completely restored to normal by removal of eserine by dialysis. The effect of some other drugs was therefore studied.

A few guinea-pigs (body-weight 600 g. to 650 g.) were injected with strychnine sulphate, morphine hydrochloride, acetylcholine bromide, and cobra venom, subcutaneously, and the C.E. activity of their blood examined by method II.

TABLE VI.

Showing the effect of drugs on the C.E. activity of blood of the animals.

Case number.	Strychnine sulphate injected.	CO ₂ before injection.	CO ₂ during convulsions.	CO ₂ 96 hours after the injection.
1	3.2 mg.	0.1308	0.1343	0.1119
2	3.2 „	0.1481	0.1429	0.1435
3	3.2 „	0.1275	0.1219	0.1229
Case number.	Morphine hydrochloride injected.	CO ₂ before injection.	CO ₂ 1 hour after the injection.	
4	31.0 mg.	0.1136	0.1190	
5	108.5 „	0.1120	0.1170	
Case number.	A.C. injected.		CO ₂ before injection.	CO ₂ 2 hours after injection.
6	3 c.c. of 1-1000 solution in 3 equal portions at 1 hour interval each.		0.10330	0.09298
7	3 c.c. of 1-100	„	0.10290	0.10680
8	3 c.c. of 1-10	„	0.14810	0.13610
Case number.	Cobra venom injected.		CO ₂ before injection.	CO ₂ after death.
9	10 mg.		0.1343	0.1343

Thus it was evident that the subcutaneous injections of the drugs had no effect on the C.E. activity of blood of the animals.

DISCUSSION.

The remarkable work of Dale and his co-workers has demonstrated the importance of A.C. as a chemical agent concerned in different physiological functions. As C.E. is concerned with the hydrolysis of A.C. it is very probable that the esterase may also play an important part in regulating the different physiological

functions of the body. The experimental work on the esterase activity of the tissues has been only recently started and the exact rôle C.E. plays in physiology is so far unexplained. Certain facts regarding the behaviour of C.E. have, however, now been recognized by several workers. Thus it is generally agreed that the C.E. values of the blood differ in different species of animals and also in different individuals of the same species. It has also been agreed that the value remains constant for the same individual. These previous observations have been confirmed by my experiments. Having confirmed the fact that the C.E. values of blood are constant for an individual over prolonged periods, attempts were made to change the physiological conditions of the animals and see if such changes made any fluctuations in the C.E. activity. It has been found that changes, such as starvation, feeding, increased temperature, loss of sleep, etc., produce but little alteration in the C.E. values of the blood of experimental animals. Administration of potent drugs, such as morphine and strychnine, also leave the esterase activity undisturbed. In fact, injections of acetylcholine itself, in comparatively large doses, fail to produce any change in the blood esterase. In all the experiments detailed above the esterase activity of the blood only has been studied. How far these different physiological variations affect the esterase activity of the tissues, it is not possible to say at the moment. The only conclusion one can draw from these results is that the esterase activity of the blood remains unchanged in the varying physiological conditions enumerated above.

SUMMARY.

1. The choline esterase activity of serum or of blood of different animal species or among individuals of the same species varies but remains fairly constant for each individual for considerably long periods.
2. The choline esterase activity of arterial and of venous blood or serum is the same and that blood taken from the animal a few minutes after its death retains its choline esterase activity.
3. Blood can be stored in the refrigerator without any appreciable loss in its choline esterase activity; dilution of blood with water reduces the choline esterase activity.
4. Sleeplessness, change in the surrounding temperature, fasting, or glucose feeding has no effect on the choline esterase content of blood.
5. Subcutaneous injection of strychnine sulphate, morphine hydrochloride, acetylcholine bromide, or cobra venom has no effect on the choline esterase content of blood.

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PREPARATION, PROPERTIES, AND PHARMACOLOGICAL ACTION OF p-AMINOBENZOYL-AMINOMETHYL- L-HYDROCOTARNINE.

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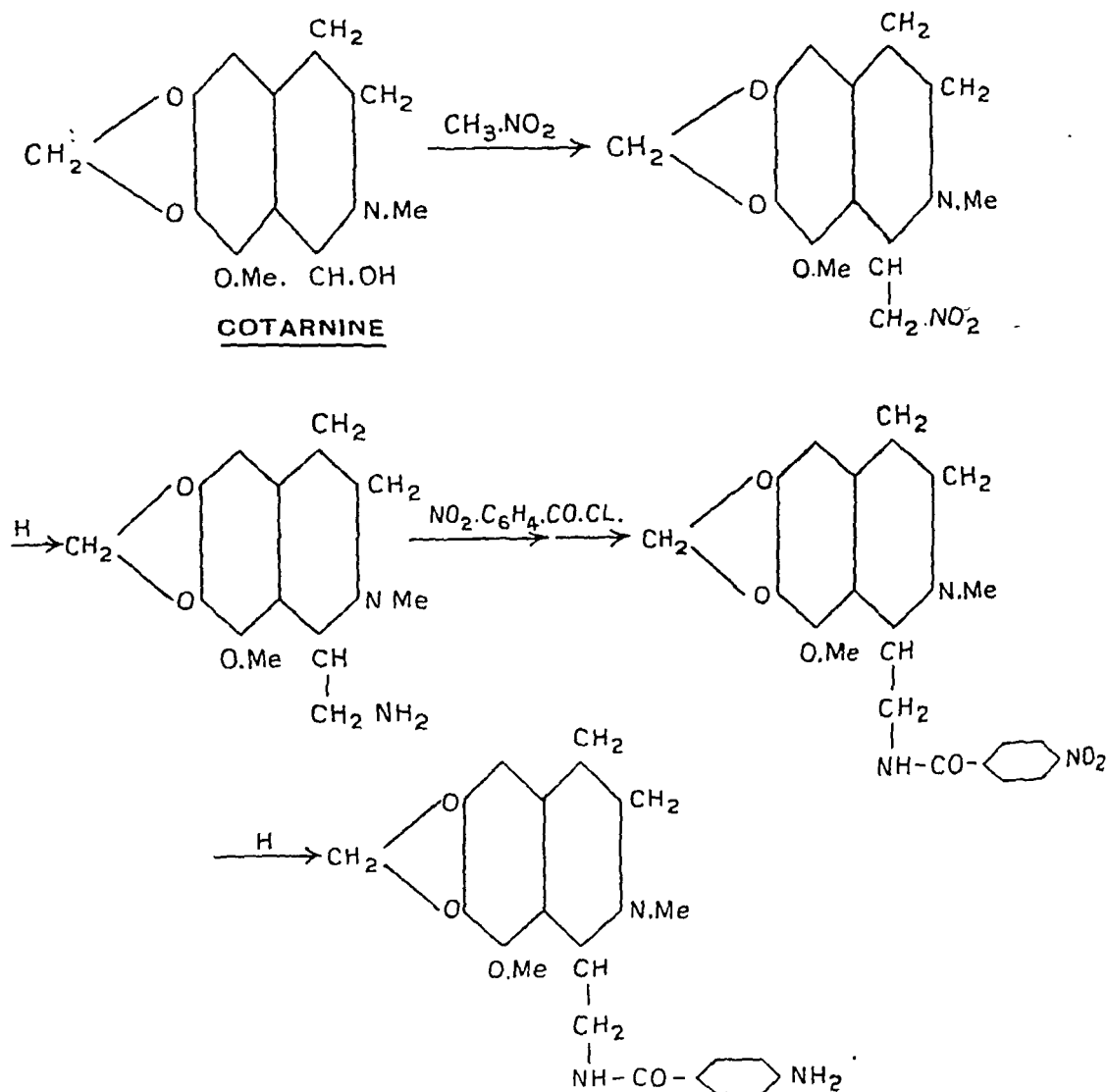
MAGIDSON and GORBOWIZKI (1935), who studied the physiological action of a number of compounds derived from aminomethyl-hydrocotarnine, reported that some of them had a powerful mydriatic action. The reaction between anhydro-cotarnino-methylamine and several aromatic nitro-acid chlorides and the reduction of the resulting compounds to the amines have recently been investigated by Dey and Lakshmikantham (1937). These products resemble closely in structure the well-known drugs of the type of novocaine and the work was undertaken in the hope that one or more members of this group might find application as useful local anæsthetics.

A number of other derivatives of cotarnine have been studied and a paper on the preparation, properties, and pharmacological action of anhydro-cotarnine-carbamide has already been published (Dey *et al.*, 1934).

Anhydro-cotarnino-methylamine, which was obtained as a yellow oil by the reduction of cotarnino-nitromethane, reacted readily with p-nitrobenzoyl-chloride in benzene solution, the crystalline hydrochloride of the nitro-base separating out almost quantitatively. The free base was prepared by prolonged treatment of the hydrochloride suspended in water with strong ammonia and the resulting

p-nitro-benzoyl-aminomethyl-hydrocotarnine was reduced to the amine by shaking with stannous chloride. Para-aminobenzoyl-aminomethyl-hydrocotarnine was a strong base and dissolved easily in cold dilute acids to form neutral solutions.

The following scheme explains the reactions involved in the preparation :—



EXPERIMENTAL.

Anhydro-cotarnine-nitromethane was prepared in quantitative yield by the method described by Hope and Robinson (1911). It crystallized from alcohol in colourless prisms melting at 129°C . It formed a sparingly soluble *hydrochloride* melting at 189°C . and a *picrate* melting at 137°C .

Anhydro-cotarnino-methylamine [Magidson and Gorbowizki (*loc. cit.*); Haworth and Perkin (1925)]. The cotarnino-nitromethane (5 grammes) was dissolved in

dry methyl alcohol (50 c.c.) containing sodium methoxide (Na, 1 gramme) and poured into a solution of 15 grammes of sodium hydroxide in 50 c.c. of water. The clear solution was heated at 60°C. on the water-bath and zinc dust (40 grammes) added in small portions during two hours. The mixture was cooled in ice, extracted thrice with ether (30 c.c. each time), the ether extract dried over potassium carbonate and the solvent removed. The pale yellow oil which remained weighed 3.1 grammes and decomposed on distillation. The *dihydrochloride* was precipitated on saturating the ethereal solution of the base with dry hydrogen chloride gas. It dissolved readily both in water and in alcohol and melted at 227°C. The *picrate* and the sparingly soluble *sulphate* melted at 200°C. and 220°C. respectively.

Para-nitrobenzoyl-aminomethyl-hydrocotarnine.—Anhydro-cotarnino-methylamine (3.5 grammes) was dissolved in dry benzene (10 c.c.) and p-nitrobenzoyl-chloride (2.6 grammes) dissolved in benzene (10 c.c.) slowly added. The hydrochloride of the base separated almost immediately as yellow crystals with considerable evolution of heat. The crude hydrochloride, after washing with benzene and then with water, and drying, weighed 4.5 grammes. It was practically insoluble in cold water but dissolved in boiling water and crystallized therefrom in colourless prismatic needles. The crystals contained one molecule of water of crystallization and melted at 234°C.

The *free base* was obtained from the powdered hydrochloride by keeping it in contact with strong ammonia for twenty-four hours. The solid changed at first into a sticky mass which slowly became granular. It was filtered, washed well with water, and crystallized from rectified spirits. It formed light yellow rhombic plates melting at 138°C. 3.1 grammes of the free base were obtained from 3.8 grammes of the hydrochloride. The base formed also a sparingly soluble *nitrate* (fern-shaped crystals) melting at 190°C. and a *picrate* melting at 138°C.

The hydrochloride as well as the free base were analysed:—

Found: Cl in dried hydrochloride, 7.39 per cent.

$C_{20}H_{21}O_6N_3.HCl$ requires Cl, 8.14 per cent.

Found: H_2O in salt, after drying at 110°/5 mm., 4.35 per cent.

$C_{20}H_{21}O_6N_3.HCl.H_2O$ requires H_2O , 3.97 per cent.

Para-aminobenzoyl-aminomethyl-hydrocotarnine.—The nitro-body (2.5 grammes) was thoroughly powdered and added very gradually to a mixture of stannous chloride (9 grammes), concentrated hydrochloric acid (25 c.c.) and a piece of granulated tin, and the whole shaken in a mechanical shaker for about ten hours. Water (125 c.c.) was added, the clear liquid was basified with excess of 25 per cent sodium hydroxide and the separated solid washed with water, dried on a porous plate and then in the desiccator, and the dry solid extracted repeatedly either with warm benzene or absolute alcohol (60°C.). The amino-body separated from the hot solvent in colourless, short prismatic rods melting at 185°C. It dissolved in dilute acids and was reprecipitated unchanged on basification. The yield was 1.2 grammes. It formed a *picrate* melting at 167°C. and gave a red dye when diazotized and coupled with β -naphthol. The acetyl derivative separated from dilute alcohol in rectangular plates melting at 135°C.

(Found: C, 64.89; H, 6.23; N, 11.09 per cent. $C_{20}H_{23}O_4N_3$ requires C, 65.04; H, 6.23; N, 11.39 per cent.)

PHARMACOLOGICAL ACTION.

Action on lower forms of life.—The lethal action of the drug was tested on *Paramæcia* cultured in the laboratory. This gives an indication regarding the action of the compound on protozoa in general, as well as bacteria. A dilution of 1 in 2,000 killed most of the *Paramæcia* in $2\frac{1}{2}$ minutes, and all of them in 6 minutes. Dilutions of 1 in 10,000 and above had no effect at all even after exposure for one hour or more. Other workers have shown that cocaine is too toxic to these protozoa in dilutions as high as 1 in 10,000, their movements being hindered, while stronger solutions stop and kill them. So the compound under investigation seems to be less toxic than cocaine in this respect.

Toxicity.—The utility of a drug is proportional to its toxicity and the determination of local anæsthetic effect is valueless unless its toxicity is also known. So we tested the toxicity of the compound on different animals. No attempt was made to correctly assess the median lethal dose, but a comparative study was made of the toxicity of the drug with that of cocaine. Injections were made subcutaneously in every case. Intravenous injections of local anæsthetics are not usually given since such a procedure is often fatal. It is known that the ratio of the toxic dose of a drug, such as procaine, to that of cocaine on subcutaneous injection bears no relation to the ratio of the intravenous toxic doses. Hence, in our opinion, comparison of the subcutaneous toxic dose of a drug with that of cocaine gives us a general idea as to its suitability for practical use.

The animals used were mice, guinea-pigs, cats, and dogs. It is known that the frog is more resistant to the toxic action of cocaine than are warm-blooded animals, and small rodents and birds are more resistant than larger mammals (*Solis-Cohen and Githens*). Our results are given below:—

TABLE I.

	M. L. D. of the compound (mg. per kilo) (approximate).	M. L. D. of cocaine (mg. per kilo) (approximate).
Mice ..	100	100
Guinea-pigs ..	80	60
Cats ..	45	25
Dogs ..	25	20

Our results show that, at any rate, the compound under investigation is not more toxic than cocaine under the same conditions.

LOCAL ANÆSTHETIC ACTION.

We studied the local anæsthetic action of the drug under three main heads :—

- (a) Blocking of conduction along frog's sciatic nerves,
- (b) Effect on rabbit's and human corneæ, and
- (c) Human wheal on intradermal injection.

(a) *Paralysis of frog's sciatic nerve*.—Two properties of the drug are measured by this method, the power to penetrate nerve sheaths and the power to paralyse sensory and motor conduction along nerve fibres.

The preparation used was the sciatic-gastrocnemius of the frog. The drug solution dissolved in saline was applied to a short length of the nerve trunk, and the muscle was kept moist by a thin layer of cotton-wool soaked in saline. The preparation was mounted in a moist chamber with glass sides. The stimulus used was a break shock from a secondary coil of an inductorium placed at a definite distance from the primary coil. and the time was measured at which the nerve failed to respond to stimulus.

The results are tabulated below :—

TABLE II.

TIME OF ONSET OF PARALYSIS OF CONDUCTION IN MINUTES.	
The drug under investigation, 1 per cent solution.	Coraine, 1 per cent solution.
48	26
12	20
28	44
3	53
35	8
43	6
42	7
47	8
36	6½
30	20
45	24

Cocaine appears to be stronger in causing paralysis of conduction along the frog's sciatic. But the variations are enormous. Sinha (1936) comparing the results obtained with different methods in assessing the local anæsthetic action of drugs found the greatest variation in the rate of paralysis of the frog's sciatic, and was of opinion that this method was unsuitable for the assay of local anæsthetics. Our results confirm his finding.

(b) *Local anæsthesia of rabbit's cornea.*—The method was to instil a few drops of a solution of the drug into one eye of the rabbit, and of an equal strength of cocaine into the other eye. The eyelashes were previously cut to prevent reflex winking. Cocaine was found to cause mydriasis in three minutes which lasted for about 30 minutes. Unlike cocaine, the drug under investigation did not cause mydriasis and was slightly irritant to the conjunctiva and cornea. The results are tabulated below :—

TABLE III.

Percentage strength of solution.	p-AMINO BENZOYL-AMINO-METHYL-L-HYDROCOTARNINE.		COCAINE.	
	Onset in minutes.	Duration in minutes.	Onset in minutes.	Duration in minutes.
1	1	15	1	Very transient.
1	1	46	13	Transient.
1	3	23	3	10
2	1	65	1	20
With adrenaline hydrochloride 1 in 50,000.				
1	3	25	1	17

(c) *On the human cornea.*—While these results show that the drug under investigation is certainly superior to cocaine in its action on the rabbit's cornea, as not only the onset of anæsthesia is quicker, but the duration of anæsthesia is also longer, we have been surprised and not a little puzzled by the contradictory results obtained on the human cornea. One to 4 per cent solutions with and without adrenaline 1 in 60,000 did not produce any appreciable loss of sensation when instilled into the human eye. The experiments were made on two of us (J. C. D. and R. K.) as well as three of the laboratory attendants, and the results were uniformly disappointing. There was some preliminary irritation with lachrymation. This passed off in a minute or two, but no further effect was noticed. The preliminary irritation was enhanced with 4 per cent solutions but no local anæsthesia followed, whereas the efficiency of cocaine in 1 to 4 per cent strengths as a surface anæsthetic in eye operations is well known. It is not understood why there should be this difference in the action of the drug under investigation on the cornea of the rabbit and that of man.

(d) *Intradermal wheal method.*—The method used was to inject 0.25 c.c. of a sterilized 0.1 per cent solution of the drug intracutaneously on the flexor surface of the forearm. A 1-c.c. syringe graduated to 0.01 c.c. was employed. The duration of the loss of sensation was measured by marking the anæsthetic area and noting the insensibility to pin pricks as compared to the surrounding area of the skin. The end point was taken when the sensitivity approached that of the normal surrounding skin. The results obtained are as follows :—

TABLE IV.

P-AMINOBENZOYL-AMINO-METHYL-L-HYDROCOTARNINE, 1 PER CENT SOLUTION.		COCAINE, 1 PER CENT SOLUTION.	
Onset in minutes.	Duration in minutes.	Onset in minutes.	Duration in minutes.
1	26	1	8
1	75	1	75
$\frac{1}{2}$	30	$\frac{1}{2}$	31
$\frac{1}{2}$	37	$\frac{1}{2}$	35
$\frac{1}{2}$	75	$\frac{1}{2}$	75

With 1 in 50,000 of adrenaline the onset of anæsthesia was almost immediate in both cases and the anæsthesia lasted for more than 100 minutes.

The local anæsthetic action of the compound under investigation as tested by the intradermal wheal method is equal to that of cocaine. It will be seen from the results that there is much less variation with the intradermal wheal method than with other methods used.

With solutions of p-aminobenzoyl-aminomethyl-l-hydrocotarnine some hyperæmia was noticed surrounding the central white area lasting for as long as three days. In one case there was some hæmorrhage into the wheal and a permanent pigmented spot has been left on the arm. Perhaps, the drug may be found useful for infiltration anæsthesia, but is certainly inferior to novocaine, because of its tendency to cause local irritation and its toxicity which approaches that of cocaine.

Adrenaline seems to enhance the local anæsthetic action of the drug tested by the 'wheal', but does not significantly increase its effect on the rabbit's cornea.

(e) *Anæsthesia of human digital nerve*.—Final tests were made on human fingers (i.e., digital nerve trunks) in combination with adrenaline, in order to test the probable clinical value of the new compound for hypodermic use. A small quantity of the solution (0.5 c.c. of 1 per cent) was injected at the root of the finger as near as possible to the digital branch of the radial nerve, the injection being made through a wheal first raised on the skin in order to make the prick of the needle painless. Anæsthesia was considered to be complete if the sensation to pin prick was abolished in the area of the distribution of the fibres of the nerve trunk, distal to the point of injection, i.e., up to the root of the nail on the lateral half of the finger.

Our results were negative, no anæsthesia being produced at the finger tip. But some swelling, redness and tenderness were noticed lasting up to 48 hours. Pain on pressure lasted as long as a week. These evidences point to the unsuitability of this compound in clinical practice.

The general pharmacological effects of the drug were studied by giving intravenous injections to anæsthetized cats and dogs, and recording the effects on various organs.

On respiration.—Intravenous injections of the drug into cats and dogs produce gradual depression of the respiratory centre leading to decrease in the amplitude of respiratory movements and marked slowing. The effects are noticed with doses as small as 5 mg., though recovery soon takes place. But with larger doses the action is more marked, and stoppage of breathing invariably occurs with toxic doses leading to circulatory failure.

On circulation.—The effects are variable. Doses as small as $2\frac{1}{2}$ mg. produce a transient rise of blood pressure. With 5 mg. this rise is rarely noticed, but a temporary diminution of blood pressure is noted. The heart *in situ* is temporarily stimulated by small doses. Even larger doses do not produce any appreciable depression of the heart.

The volume of the spleen tends to fall with small doses, but often there is a marked rise subsequently with great improvement in the automatic movements. The isolated spleen perfused in a Dixon's apparatus shows a preliminary contraction followed by dilatation of the blood vessels as noticed by the outflow of the perfusate. The kidney volume also shows a slight increase.

Plain muscles like the intestine and uterus show increase in tone and rhythmicity. The effect on the uterus is not as marked as with cotarnine itself.

SUMMARY.

1. The preparation and chemical properties of *p*-aminobenzoyl-aminomethyl-l-hydrocotarnine is described.
2. *p*-aminobenzoyl-aminomethyl-l-hydrocotarnine is a strong base dissolving easily in cold dilute acids to form neutral solutions.
3. The toxicity of *p*-aminobenzoyl-aminomethyl-l-hydrocotarnine is not greater than that of cocaine.

4. The local anæsthetic action of the drug was studied by different methods and compared with cocaine. It was found to produce corneal anæsthesia in the rabbit almost instantaneously, and the effect lasted much longer than with cocaine. But strangely enough it had no action on the human cornea. The local anæsthetic action as judged by the intradermal wheal method was equal to that of cocaine and was enhanced by the addition of adrenaline.

5. Small doses given intravenously to dogs and cats had little effect on circulation and respiration. Sometimes there was a transient stimulation of the heart or rise of blood pressure. With larger doses the respiration was slowed and depressed, and finally failed.

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RED CELLS, HÆMOGLOBIN, COLOUR INDEX, SATURATION INDEX, AND VOLUME INDEX STANDARDS.

Part II.

NORMAL INDIAN WOMEN : A STUDY BASED ON THE EXAMINATION OF 101 WOMEN.

BY

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IN a previous paper (Sokhey *et al.*, 1937) attention was drawn to the lack of accurate data for normal standards for blood, the figures usually given in textbooks being based on a few examinations carried out a long time ago, when the methods were not so accurate. During the last ten years a number of workers, chiefly Osgood (1926, 1927), Wintrobe and Miller (1929), Wintrobe (1930), Price-Jones (1931), and Haden (1933), have published accurate data for white Americans and Europeans. We have carried out similar studies for normal Indian men and women. Data for normal Indian men have already been published (Sokhey *et al.*, *loc. cit.*), and in this paper we present the result of a study of 101 normal Indian women.

Until a short summary of our early results appeared in the report of the Haffkine Institute for the year 1929 (Sokhey, 1929), no data for normal Indian women were available. Even for European women no recent accurate data were available until Osgood and Haskins (1927) published their study of 100 white American women. Since then more data have been published in America and Europe by other workers, for example, Wintrobe (1930) for 50 women, Price-Jones (1931) for 100 women, and Haden (1933) for 30 women. Napier and Das

Gupta (1935) have published the results of a study based on the examination of 17 normal Indian coolie women. But they remark 'although we have used the word "normal" there is strong evidence to suggest that in these coolies the erythron is not in a state of physiological equilibrium'.

The one hundred and one women examined by us were medical students or nurses from the neighbouring Medical College and hospitals. Our subjects were between 18 and 30 years of age except five, three of whom were 16 and two 17. This age group was selected so that our results may be comparable to the only extensive study published at the time (Osgood and Haskins, *loc. cit.*).

All our subjects belonged to the Bombay Presidency. Some of them were vegetarians, while others were on mixed diet. And as we have pointed out in our previous paper (*loc. cit.*) this matter of diet did not constitute an important difference. Both groups subsisted more or less on the same sort of diet except that some ate a little mutton or eggs now and then. Both groups consumed milk. None of the subjects was pregnant at the time of examination and no attention was paid to their menstrual cycle.

We do not describe the methods used for this study, as they have been already fully described in our previous paper (*loc. cit.*).

TABLE I.

Blood findings in one hundred and one normal young women.

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
1	20	V	3.85	11.60	15.07	30.04	39.01	1.04	0.96	1.08
2	20	V	3.81	11.86	15.56	31.80	41.74	1.07	1.03	1.04
3	27	M	4.63	11.82	12.76	34.13	36.84	0.88	0.91	0.97
4	30	M	4.53	12.94	14.28	35.93	39.67	0.98	0.98	1.01
5	27	M	4.78	12.84	13.43	36.81	38.50	0.92	0.95	0.97
6	25	V	3.65	10.46	14.33	31.12	42.63	0.98	1.05	0.94
7	23	M	4.50	11.00	12.22	33.82	37.58	0.84	0.93	0.91
8	28	V	4.53	14.28	15.76	40.62	44.83	1.08	1.10	0.98
9	22	V	4.37	13.40	15.33	37.79	43.24	1.05	1.06	0.99
10	22	M	4.02	11.07	13.77	34.77	43.24	0.95	1.06	0.89

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE I—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmoglobin, grammes per 100 c.c.	Hæmoglobin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
11	20	V	4.48	13.83	15.44	35.38	39.49	1.06	0.97	1.09
12	25	V	4.24	11.45	13.50	34.31	40.44	0.93	1.00	0.93
13	30	M	4.16	12.18	14.64	34.66	41.65	1.01	1.03	0.98
14	22	M	4.62	12.54	13.57	37.22	40.28	0.93	0.99	0.94
15	21	M	4.03	12.50	15.51	34.32	42.58	1.07	1.05	1.02
16	24	M	4.15	13.39	16.13	36.26	43.69	1.11	1.08	1.03
17	26	M	4.68	13.07	13.96	37.12	39.67	0.96	0.98	0.98
18	29	M	4.37	13.08	14.97	33.80	38.68	1.03	0.95	1.08
19	24	M	4.31	11.78	13.67	37.50	43.50	0.94	1.07	0.88
20	28	M	4.37	12.60	13.79	35.85	39.22	0.95	0.97	0.98
21	29	M	4.35	12.61	14.49	35.22	40.48	1.00	1.00	1.00
22	22	M	3.50	10.91	15.59	30.85	44.07	1.07	1.09	0.99
23	22	V	4.24	12.68	14.95	37.62	44.38	1.03	1.09	0.94
24	21	M	4.00	10.97	13.71	32.23	40.28	0.94	0.99	0.94
25	24	M	4.27	11.48	13.44	35.04	41.03	0.93	1.01	0.92
26	26	M	4.20	11.62	13.83	36.07	42.94	0.95	1.06	0.90
27	24	M	4.10	11.57	14.11	34.49	42.06	0.97	1.04	0.94
28	20	M	4.94	14.57	14.75	36.58	37.02	1.01	0.91	1.11
29	25	V	4.27	13.41	15.70	35.26	41.29	1.08	1.02	1.06
30	24	M	4.41	11.43	12.96	35.79	40.58	0.89	1.00	0.89
31	21	V	4.65	13.69	14.72	38.20	41.07	1.01	1.01	1.00
32	23	M	5.21	12.33	11.83	40.15	38.53	0.81	0.95	0.86
33	18	M	4.29	12.32	14.36	35.20	41.02	0.99	1.01	0.98
34	25	M	4.41	13.50	15.31	36.93	41.87	1.05	1.03	1.02
35	22	V	4.53	13.78	15.21	33.93	37.45	1.05	0.92	1.13

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE I—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmoglobin, grammes per 100 c.c.	Hæmoglobin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
36	19	M	4.60	14.85	16.14	40.00	43.47	1.11	1.07	1.04
37	22	M	4.93	14.65	14.86	41.82	42.41	1.02	1.04	0.98
38	24	M	4.18	11.66	13.95	34.70	41.50	0.96	1.02	0.91
39	23	M	4.66	14.13	15.16	33.71	36.16	1.04	0.89	1.17
40	22	M	5.20	14.77	14.20	41.24	39.66	0.98	0.98	1.00
41	23	M	4.34	13.34	15.37	32.31	37.21	1.06	0.92	1.15
42	18	M	4.75	14.29	15.04	39.01	41.06	1.03	1.01	1.02
43	21	M	4.93	15.01	15.22	40.36	40.94	1.05	1.01	1.04
44	26	V	4.65	13.58	14.60	37.22	40.01	1.00	0.99	1.02
45	23	M	4.13	11.43	13.84	28.40	34.38	0.95	0.85	1.12
46	19	M	5.20	14.22	13.67	40.37	38.82	0.94	0.96	0.98
47	28	M	4.49	13.18	14.68	37.02	41.23	1.01	1.02	0.99
48	17	M	4.57	14.03	15.35	33.77	36.95	1.06	0.91	1.16
49	22	M	4.55	13.65	15.00	39.32	43.21	1.03	1.06	0.97
50	20	M	4.33	13.07	15.09	35.92	41.49	1.04	1.02	1.02
51	26	M	4.18	13.36	15.98	31.34	37.49	1.10	0.92	1.19
52	27	M	4.56	13.78	15.11	37.74	41.38	1.04	1.02	1.02
53	28	V	4.54	11.79	12.98	33.46	36.84	0.89	0.91	0.98
54	20	M	4.92	13.16	13.37	33.93	34.48	0.92	0.85	1.08
55	18	M	4.44	13.73	15.46	39.37	44.34	1.06	1.09	0.97
56	19	M	4.67	13.63	14.59	38.13	40.83	1.00	1.01	1.00
57	19	V	4.36	11.46	13.14	29.18	33.47	0.90	0.82	1.10
58	21	M	4.58	13.02	14.21	38.07	41.57	0.98	1.02	0.95
59	21	M	4.97	14.46	14.55	40.00	40.24	1.00	0.99	1.01
60	19	M	4.67	12.42	13.30	36.08	38.63	0.91	0.95	0.96

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE I—*contd.*

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmo-globin, grammes per 100 c.c.	Hæmo-globin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
61	23	M	5.35	14.20	13.27	36.32	33.94	0.91	0.84	1.09
62	18	M	4.88	13.72	14.06	37.65	38.59	0.97	0.95	1.02
63	18	M	4.54	12.54	13.81	35.14	38.69	0.95	0.95	1.00
64	21	M	4.37	12.70	14.53	32.46	37.14	1.00	0.91	1.09
65	23	V	4.31	13.17	15.28	37.24	43.20	1.05	1.06	0.99
66	20	M	3.97	13.02	16.40	31.17	39.24	1.13	0.97	1.16
67	18	M	4.60	13.82	15.02	40.49	44.00	1.03	1.08	0.95
68	23	V	4.52	13.45	14.88	37.45	41.43	1.02	1.02	1.00
69	25	V	4.82	13.90	14.42	38.25	39.68	0.99	0.98	1.01
70	25	M	4.23	13.00	15.37	34.75	41.07	1.06	1.01	1.04
71	21	M	4.51	13.45	14.91	38.58	42.77	1.03	1.03	0.97
72	22	M	4.28	12.45	14.54	34.48	40.28	1.00	0.99	1.01
73	18	V	3.96	11.30	14.27	30.20	38.14	0.98	0.94	1.04
74	21	V	4.44	13.30	14.98	38.27	43.09	1.03	1.06	0.97
75	24	M	4.45	12.99	14.60	37.44	42.07	1.00	1.04	0.97
76	16	V	4.08	12.18	14.93	32.73	40.11	1.03	0.99	1.04
77	20	V	4.32	13.80	15.97	40.38	46.73	1.10	1.15	0.95
78	24	V	4.66	14.49	15.55	42.06	45.12	1.07	1.11	0.96
79	28	V	4.70	10.72	11.40	33.44	35.57	0.78	0.88	0.89
80	16	V	4.48	14.23	15.88	38.33	42.77	1.09	1.05	1.04
81	20	V	3.90	11.97	15.35	37.20	47.68	1.06	1.17	0.90
82	22	M	4.31	13.24	15.36	35.66	41.36	1.06	1.02	1.04
83	18	M	4.22	13.23	15.68	36.17	42.85	1.08	1.05	1.02
84	26	M	4.70	13.55	14.41	39.36	41.88	0.99	1.03	0.96
85	24	M	4.68	13.46	14.38	39.29	41.99	0.99	1.03	0.96

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE I—concl'd.

Serial number.	Age.	Diet.	Red cell count, millions per c.mm.	Hæmoglobin, grammes per 100 c.c.	Hæmoglobin coefficient.	Cell volume, c.c. per 100 c.c.	Cell volume coefficient.	Colour index.	Volume index.	Saturation index.
86	18	M	5.11	16.01	15.67	44.67	43.71	1.08	1.08	1.00
87	27	M	4.93	13.74	13.62	39.90	40.48	0.96	1.00	0.96
88	19	M	4.54	11.31	12.46	37.83	41.66	0.86	1.02	0.83
89	24	M	4.57	13.78	15.08	40.42	44.23	1.04	1.09	0.95
90	21	M	4.53	12.80	14.13	36.55	40.33	0.97	0.99	0.98
91	25	M	4.64	13.06	14.07	35.49	38.24	0.97	0.94	1.03
92	24	M	4.66	13.73	14.73	39.70	42.60	1.01	1.05	0.97
93	23	M	4.77	14.08	14.76	38.62	40.49	1.01	1.00	1.02
94	23	M	4.29	12.45	14.51	35.71	41.61	1.00	1.02	0.97
95	17	M	4.44	13.28	14.95	37.54	42.27	1.03	1.04	0.99
96	25	M	4.42	13.25	14.99	37.89	42.86	1.03	1.05	0.98
97	22	M	4.17	13.00	15.59	35.09	42.07	1.07	1.04	1.03
98	23	M	4.11	12.57	15.29	31.88	38.79	1.05	0.95	1.10
99	30	M	4.56	13.52	14.82	33.95	37.22	1.02	0.92	1.11
100	20	M	4.37	13.06	14.94	36.00	41.19	1.03	1.01	1.02
101	16	V	4.58	13.55	14.79	38.15	41.65	1.02	1.03	0.99
AVERAGE	22.5	..	4.47	12.99	14.55	36.27	40.61	1.00	1.00	1.00

In the diet column, V stands for vegetarian diet and M for mixed diet.

RED CELL COUNTS.

Our average for 101 women is 4,465,000 per cubic millimetre. The values range from 3.50 to 5.35 millions. The mean (4.465 ± 0.022) and the median (4.495 ± 0.028) are very close. The standard deviation is 0.328 ± 0.016 and the coefficient of variation is 7.340 ± 0.0351 . The significant variation is 4.14 — 4.79, and 73 per cent of our subjects fall within this range. The frequency distribution of red cell counts in our 101 women subjects is shown in Fig. 1.

The figure for average normal red cell count for women usually given in European and American textbooks is 4.5 millions. Osgood and Haskins (*loc. cit.*) obtained an average of 4.80 millions by the study of 100 white American women (ages 18 to 30) from western United States and Wintrobe (1930) an average of 4.93 millions based on the study of 50 white American women (ages 17 to 30) from southern United States. Haden (1922, 1933) found an average of 4.38 for 30 women and 4.26 for 12 women from middle-west U. S. A. Price-Jones (1931) obtained an

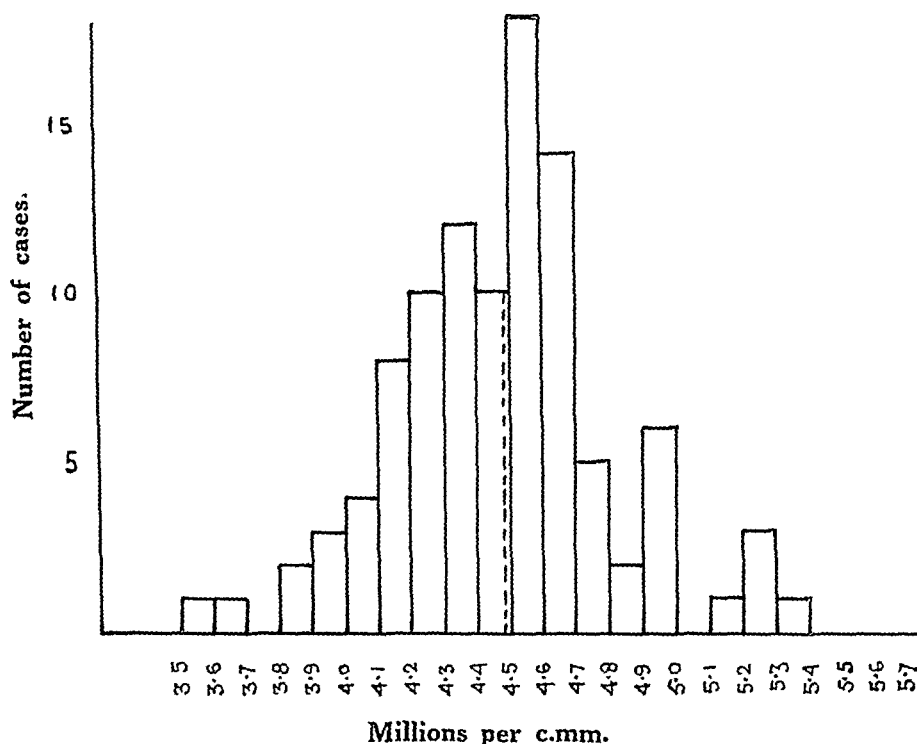


FIG. 1.—Red cell counts in 101 women.

average of 5.01 millions for 100 women in England. Wintrobe (1933) gives an average of 4.85 millions for 369 women subjects from figures available in literature from Europe and America. Sachs, Levine and Fabian (1935) found an average of 4,508,500 for 100 normal white women in central U. S. A. between the ages of 20 and 30 years. All these averages except those obtained by Haden are higher than the average obtained by us, viz., 4.47 millions.

HÆMOGLOBIN CONTENT.

Our average is 12.99 g. per 100 c.c. of blood corresponding to an oxygen capacity of 17.41 c.c. per 100 c.c. of blood. The hæmoglobin values range from 10.46 g. to 16.01 g. The mean (12.993 ± 0.074) and the median (13.190 ± 0.093)

are quite close. The standard deviation is 1.102 ± 0.052 and the coefficient of variation is 8.472 ± 0.404 . The significant variation thus becomes 11.89–14.10, and covers 67 per cent of our subjects. Fig. 2 is a histogram giving the frequency distribution of hæmoglobin in our 101 women subjects.

Osgood and Haskins (*loc. cit.*) give an average of 14.67 g. per 100 c.c. of blood from reliable hæmoglobin estimations reported in literature for three different series of subjects, totalling in all 64 women between the ages of 18 and 30 years. The averages for these different series varied from 12.82 g. to 15.11 g. per 100 c.c. They also found reports of 143 examinations of women in which the age was not stated or in which the methods used for estimation were of doubtful

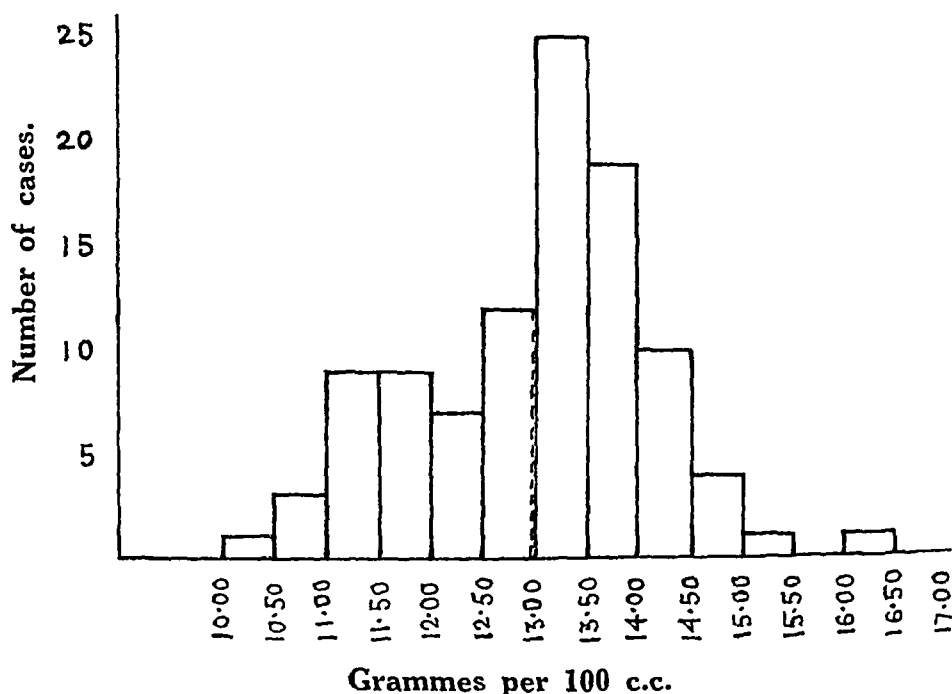


FIG. 2.—Hæmoglobin in 101 women.

accuracy. These examinations give an average of 12.88 g. Osgood and Haskins' (*loc. cit.*) average for 100 women of ages between 18 and 30 years is 13.69 g. per 100 c.c. Wintrobe (1930) found an average of 13.76 g. on a study of 50 women, between the ages of 17 and 30 years. Haden (1933) obtained an average of 13.37 g. per 100 c.c. for 30 women. Sachs, Levine and Fabian (*loc. cit.*) found an average of 12.96 g. for 100 women between the ages of 20 and 30 years, estimating the hæmoglobin by Wong's iron method.

The hæmoglobin figures given in the previous paragraph were determined by different workers by colorimetric methods, while Haden (1922) and Price-Jones (1931) have reported determinations made by oxygen capacity methods. Haden using the van Slyke method obtained an average of 12.93 g. $\left(\frac{17.33 \text{ c.c.}}{1.34}\right)$ of hæmoglobin per 100 c.c. of blood for a series of 12 normal white American women

between the ages of 20 and 40 years from Kansas City. Price-Jones made his determinations by the Haldane blood gas apparatus and obtained an average value of 13.5 g. $\left(\frac{18.1 \text{ c.c.}}{1.34}\right)$ hæmoglobin per 100 c.c. of blood for a series of 100 healthy London women of the average age of 24 years. Price-Jones also examined a series of 100 men in London by the same method, but finding his average of 14.5 g. for men to be lower than the hæmoglobin averages of 15.5 g. and 15.4 g. obtained by Haden and Dill in America, he went to Boston and there compared his Haldane blood gas method with the van Slyke method. He found little or no difference between the two methods. He therefore attributed the higher American figures to a possible polycythæmia resulting from a slow carbon monoxide poisoning caused by the extensive motoring habit of the Americans. It is natural to assume that the American women would be subject to the same conditions as the men in that country and would tend to have a higher hæmoglobin figure than women in London for the same reason. Thus, the average hæmoglobin figure for American white women would be markedly higher than Price-Jones' average of 13.5 g. for London women. But it is not so. Haden's average for American women is actually lower (12.93 g.). We do not think Price-Jones' explanation holds good. Even the figures for American women based on colorimetric methods which, as was pointed out in our previous paper, usually give a somewhat higher figure than the van Slyke oxygen capacity method, are not definitely higher than Price-Jones' average for London women.

It is to be noted that our series of 101 women were examined by the van Slyke oxygen capacity method, and the average of 12.99 g. hæmoglobin per 100 c.c. of blood compares closely with Haden's average of 12.93 g. obtained by the same method. Though the number of subjects examined by Haden is small, the similarity of results is significant because of the similar correspondence in the hæmoglobin averages of a much larger series of men examined by the van Slyke method by us and other workers (Sokhey *et al.*, 15.37 g., Haden, 15.34 g., and Dill, 15.38 g.). Only further work can show whether the observed correspondence is a mere coincidence or whether there is some further explanation.

HÆMOGLOBIN COEFFICIENT AND COLOUR INDEX.

For assessing the value of the hæmoglobin content of a given individual comparison with a normal hæmoglobin standard is necessary. The normal standard is taken to be the average number of grammes of hæmoglobin per hundred cubic centimetres of blood calculated to a count of 5 millions red cells per cubic millimetre in the average healthy person of the same age and sex group. Osgood (1926) suggests the term 'hæmoglobin coefficient' to replace the cumbersome expression 'the number of grammes of hæmoglobin per hundred cubic centimetres of blood calculated to a red cell count of 5 millions per cubic millimetre'.

The actual hæmoglobin content in grammes per hundred cubic centimetres of blood of the individual is converted into a percentage of the normal hæmoglobin coefficient of the same age and sex group. Also the red cell count of the individual is converted into a percentage of a count of 5 millions taken as 100 per cent. The colour index is then calculated by dividing the percentage of hæmoglobin by the percentage of red cells.

The figure of 5 millions as 100 per cent of red cells in colour and other index determinations is used for convenience in calculation and as Osgood (1926-27) says 'any other figure would do, if it were generally agreed upon'. Five millions is the generally accepted figure, but it must be clearly understood that it is not the average red cell count for either normal men or women.

Our average hæmoglobin coefficient for the 101 women is 14.55 g. The figures range from 11.40 g. to 16.40 g. The mean is 14.553 ± 0.065 and the median 14.710 ± 0.081 . Standard deviation is 0.964 ± 0.046 and the coefficient of variation is 6.622 ± 0.319 . The significant variation therefore is 13.59 — 15.52, and 71 per cent of our subjects fall within this range. The frequency distribution of our hæmoglobin coefficients is shown in Fig. 3.

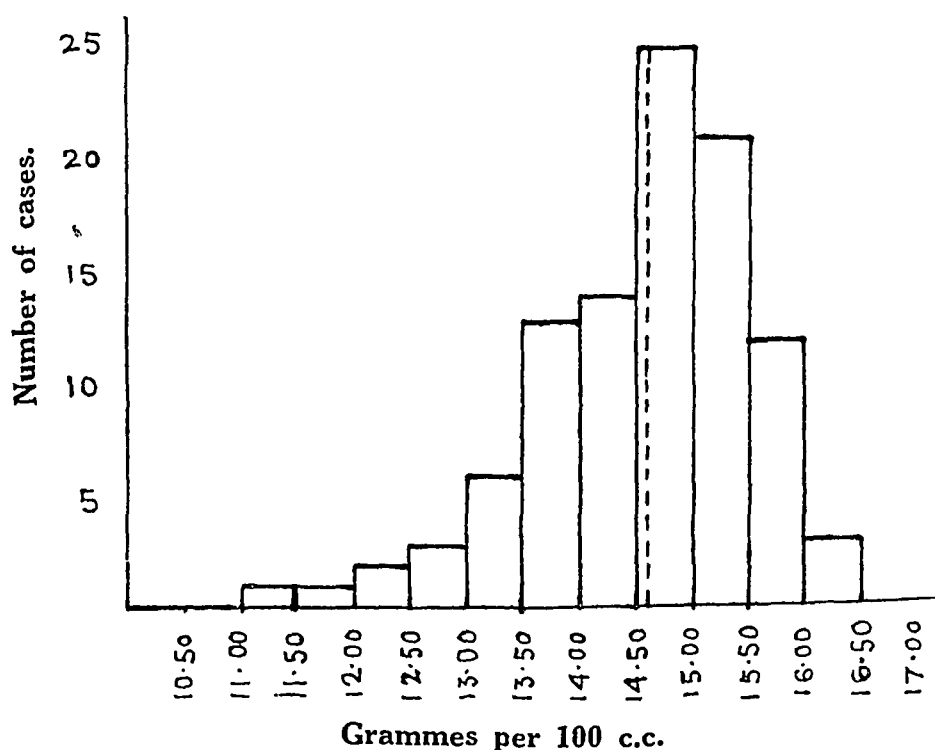


FIG. 3.—Hæmoglobin coefficients in 101 women.

Our average colour index is 1.00, the figures ranging between 0.78 and 1.13. Frequency distribution of our colour indices is shown in Fig. 4. The mean (1.003 ± 0.004) and the median (1.016 ± 0.005) are close. Standard deviation is 0.0653 ± 0.003 and the coefficient of variation is 6.489 ± 0.349 . The significant variation therefore is 0.94 — 1.07, and 71 per cent of our subjects are within this range.

Osgood and Haskins (*loc. cit.*) find the hæmoglobin coefficient to be 14.29 g. for their series of 100 women, and Wintrobe (1930) obtained a figure of 13.97 g. for 50 women. Wintrobe (1933) collected data for 369 women from the literature; for

this series the average hæmoglobin coefficient is 14.33 g. Haden (1933) obtained for a series of 30 women a hæmoglobin coefficient of 15.26 g. The hæmoglobin coefficient for our subjects is 14.55 g.

The situation regarding the hæmoglobin coefficient in the case of women is precisely the same as was noted in the case of men. It does not seem that we are anywhere near having a common figure for normal hæmoglobin coefficient. It is not clear whether such a thing is possible. But the situation makes it imperative that further accurate studies should be made in as many different places as possible.

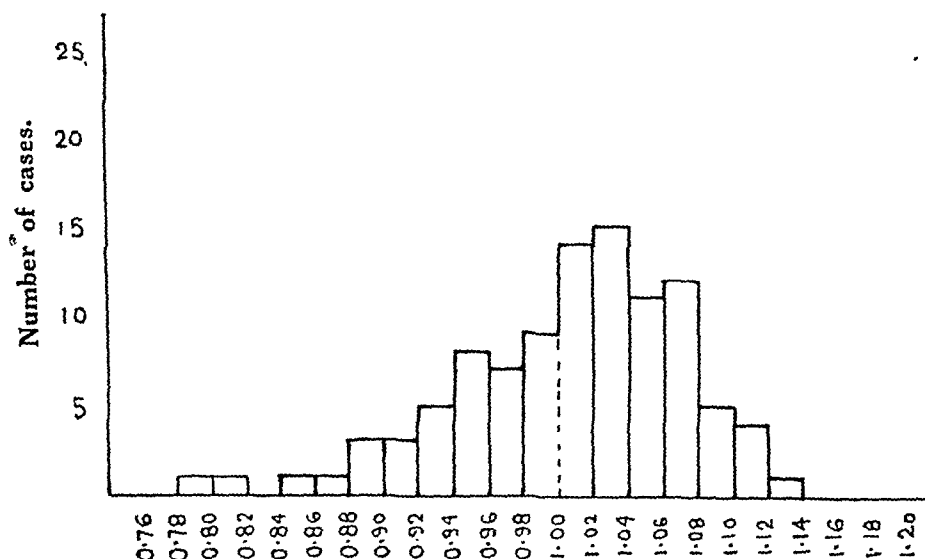


FIG. 4.—Colour indices in 101 women.

In the meantime we commend Haden's (1933) suggestion that for each area the mean values for hæmoglobin and the red cell count should be determined and these used for reporting to clinicians in that area the hæmoglobin percentage and the colour and saturation indices.

TOTAL CELL VOLUME.

Our average figure for the total cell volume is 36.27 c.c. per 100 c.c. of blood: its frequency distribution is shown in Fig. 5. The values obtained range from 28.40 c.c. to 44.67 c.c. The mean (36.269 ± 0.205) and the median (36.450 ± 0.256) are close. The standard deviation is 3.048 ± 0.145 and the coefficient of variation is 8.391 ± 0.399 . The significant variation is 33.22 — 39.32, and covers 70 per cent of our subjects.

It should be noted that our determinations were done on samples of blood to which neutral potassium oxalate (2 mg. per c.c. of blood) was added to prevent coagulation. We have not corrected for shrinkage the figures obtained by actual experiment.

Osgood and Haskins (*loc. cit.*) obtained an average value of 41.04 c.c. per 100 c.c. of blood for their series of 100 women. This figure is also uncorrected for shrinkage. Wintrobe (1930) found for 50 women an average value of 38.04 c.c. per 100 c.c. of blood*. For data which he collected from literature for 369 women, Wintrobe (1933) gives an average cell volume of 38.35 c.c. (i.e., the actual figure given is 41.8 c.c., but this is obtained after correction for shrinkage of 8.20 per cent). It will be seen that our average cell volume figure of 36.27 c.c. is the lowest of all; however it must also be remembered that our average red cell count is also the lowest.

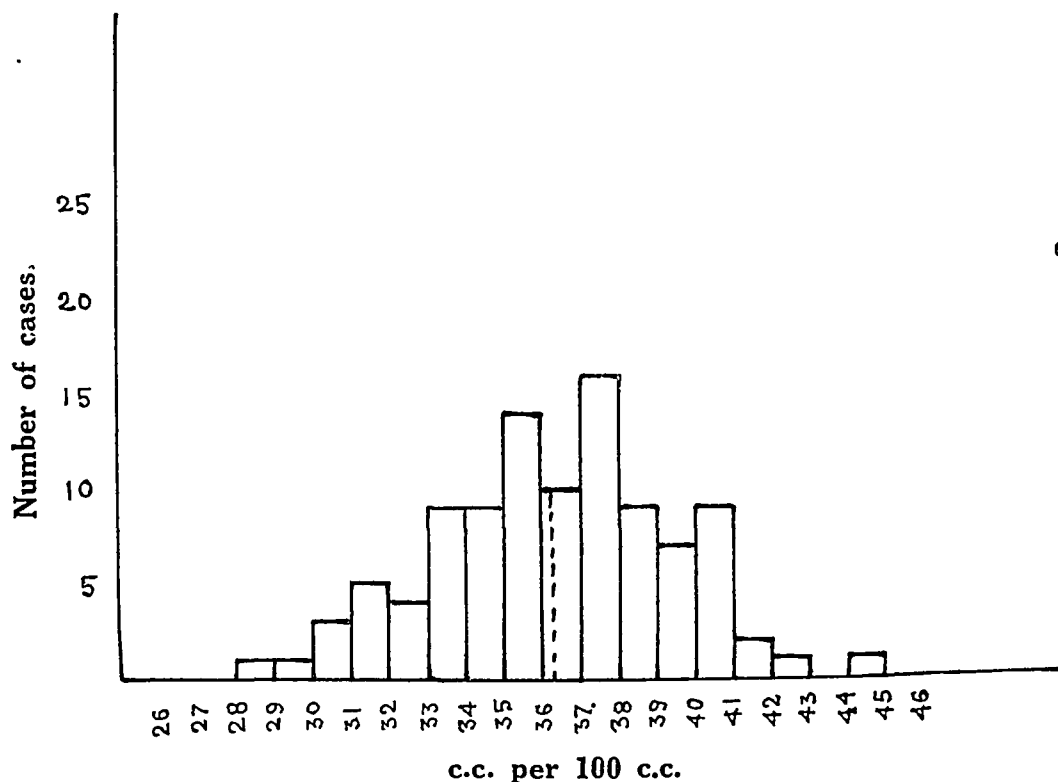


FIG. 5.—Cell volume in 101 women.

Attention is drawn to the different values for shrinkage given by different workers. We refer the reader to the experiments reported by us in our previous paper on normal standards for healthy young Indian men. We obtained a value for shrinkage of 5.75 per cent as compared to Osgood's (1926) 3.5 per cent, and Wintrobe and Miller's (*loc. cit.*) 3.7 per cent. Later, Wintrobe gave a value of 5.75 per cent in 1931 and a value of 8.2 per cent in 1932.

* The figures actually given by Wintrobe (1930) are those corrected by an addition of 3.7 per cent to the cell volumes determined in each case to allow for shrinkage resulting from the addition of oxalate to blood (10 mg. to 5 c.c. blood). We have reconverted the average figure of 39.5 c.c. of this series to 38.04 c.c. to render possible a comparison between his figures and ours, which are not corrected for shrinkage. Later, Wintrobe (1932) has changed his correction for shrinkage from 3.7 per cent to 8.2 per cent, and consequently (1933) gave 41.5 c.c. as his mean average for women instead of the original 39.5 c.c.

If the shrinkage is constant and the volume index calculation is based on standards determined by the potassium oxalate method, the indices will be absolutely correct. When absolute figures for cell volume are needed, they could be obtained by the use of a factor calculated from the measurement of the extent of shrinkage caused by the use of potassium oxalate.

VOLUME COEFFICIENT AND VOLUME INDEX.

In our previous paper we pointed out the value of volume index determination. This index is easily determined and is a good indication of the average size of the individual red cells.

Just as the colour index expresses the ratio of the amount of hæmoglobin per red cell of the individual to that of the average normal, similarly the volume index expresses the ratio of the volume of the red cell of the individual to that of the average normal. It is determined in the same way as the colour index with the difference that cell volume percentage is substituted for hæmoglobin percentage, i.e., the volume of the cells expressed as percentage of the average normal cell volume is divided by the percentage of red cells. The average volume of cells in 100 c.c. of blood of healthy persons of the same sex and age group calculated to red cell count of 5 millions is the normal standard, i.e., normal volume coefficient, and is taken as 100 for the calculation of the percentage of red cell volume. For the determination of percentage of red cells the figure of 5 millions is taken as 100 per cent for reasons already given.

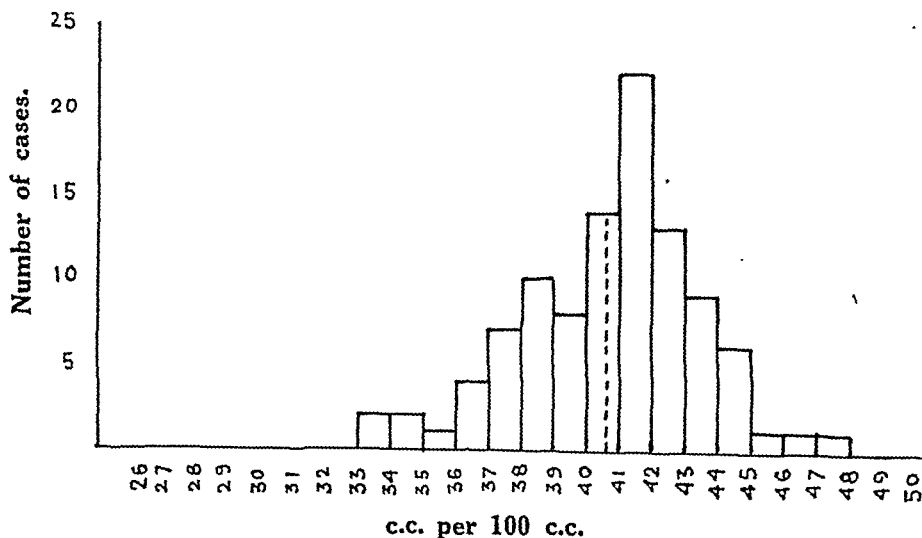


FIG. 6.—Cell volume coefficients in 101 women.

Our average figure for total cell volume for 101 women being 36.27 c.c. per 100 c.c. of blood and our average red cell count for the same subjects being 4.465 millions per c.mm., our average volume coefficient works out to be 40.61 c.c. The frequency distribution of the volume coefficients is shown in Fig. 6. The values

range from 33.47 to 47.68, the mean and the median being 40.615 ± 0.179 and 41.114 ± 0.224 , respectively. The standard deviation is 2.667 ± 0.127 and the coefficient of variation is 6.552 ± 0.313 . The significant variation thus is 37.95 — 43.29, and covers 71 per cent of our subjects.

Osgood and Haskins (*loc. cit.*) obtained a volume coefficient of 42.8 and Wintrobe (1930) 38.58*. The data for 369 women (European and white American) collected from literature by Wintrobe (1933) yields a cell volume coefficient of 39.54*.

Osgood and Haskins (*loc. cit.*) get a higher average volume coefficient (42.8 c.c.) for women than the average volume coefficient (40.8 c.c.) for men obtained by one of them (Osgood, 1926). They found the difference to be significant. They say 'this result was unexpected, and we have been unable to find any reason why young women should have larger cells than young men This apparent difference should be confirmed by workers on a large series of subjects'. Wintrobe and Miller (*loc. cit.*) and Wintrobe (1930) give a figure of 40.0 c.c. for men and 40.3 c.c. for women. But these values are not strictly comparable because of the different proportions of potassium oxalate used in the two series of determinations. Our results (40.83 c.c. for men and 40.61 c.c. for women) do not confirm the finding of Osgood. Our figures for the average volume coefficients for men and women are almost the same. This would tend to show that the cells of men and women are similar in size. Our observation confirms Price-Jones' (1933) finding.

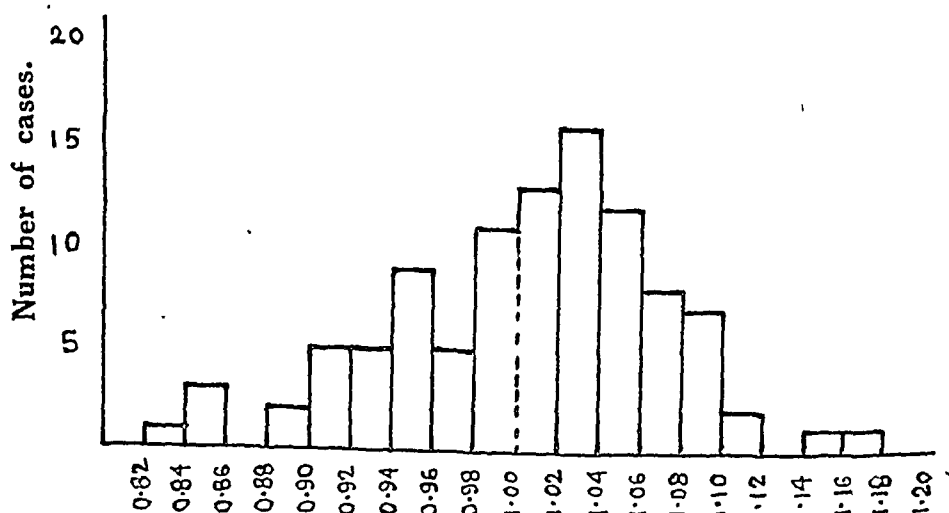


FIG. 7.—Volume indices in 101 women.

Our average volume index is 1.00, the values ranging from 0.82 to 1.17. The frequency distribution of our volume indices is shown in Fig. 7. The mean and the median are 1.002 ± 0.004 and 1.014 ± 0.006 , respectively. The standard deviation is 0.065 ± 0.003 and the coefficient of variation is 6.443 ± 0.306 . The significant variation is thus 0.94 — 1.07, and covers 75 per cent of our subjects

* This figure is calculated by allowing for shrinkage (see footnote on page 734).

SATURATION INDEX.

Saturation index expresses the ratio between the concentration of hæmoglobin per unit volume of cells in a particular subject and the average concentration of hæmoglobin per unit volume of cells in normal subjects of the same age and sex groups. It is determined by dividing the colour index by the volume index. This index is a better indication than the colour index of an increase or decrease of hæmoglobin in blood cells in disease.

Our average saturation index is 1·00, the values ranging between 0·83 and 1·19. The frequency distribution of our saturation indices is indicated in Fig. 8. The mean is $1\cdot001 \pm 0\cdot005$ and the median is $0\cdot999 \pm 0\cdot006$. The standard deviation is $0\cdot0683 \pm 0\cdot003$ and the coefficient of variation $6\cdot774 \pm 0\cdot322$. The significant variation thus becomes 0·93 — 1·07, and 72 per cent of our subjects fall within this range.

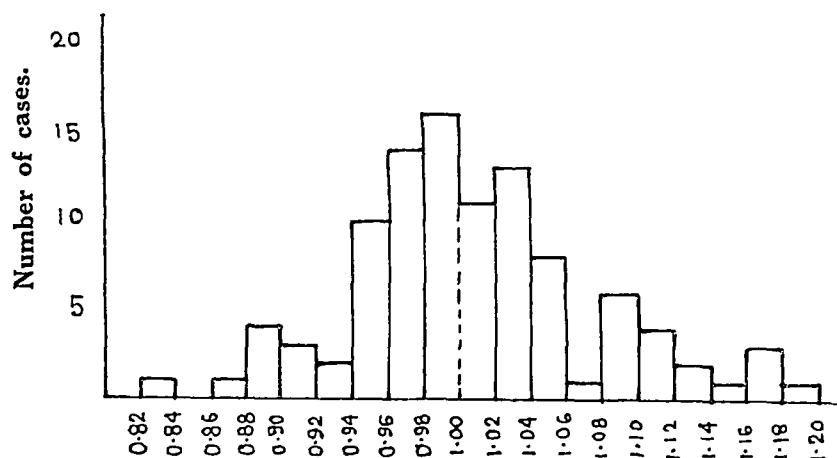


FIG. 8.—Saturation indices in 101 women.

METHOD OF CALCULATING RESULTS.

We give here an illustration of how the indices are calculated :—

Case 59 of our series gave the following results on examination of the blood :—

Red cell count	4·97 millions per c.mm.
Hæmoglobin	14·46 grammes per 100 c.c.
Cell volume	40·00 c.c. per 100 c.c.

For converting these figures into percentages for the purpose of calculation of the indices, a red cell count of 5 millions and the 'coefficients' are taken as 100, as has already been explained on page 731.

The average hæmoglobin content of 12·99 g. per 100 c.c. for our series of normal Indian women (average red cell count of 4·465 millions per c.mm.) calculated to a red cell count of 5 millions, gives 14·55 g. as the average 'hæmoglobin coefficient' for our series $\left(\frac{12\cdot99 \times 5}{4\cdot465} = 14\cdot55 \right)$.

Similarly, the average cell volume of 36.27 c.c. per 100 c.c. for our series, calculated to a red cell count of 5 millions, gives 40.61 c.c. as the average volume coefficient for our series $\left(\frac{36.27 \times 5}{4.465} = 40.61\right)$.

Thus: the red cell count of 4.97 millions becomes 99.4 per cent $\left(\frac{4.97 \times 100}{5}\right)$.

The hæmoglobin content of 14.46 g. becomes 99.4 per cent $\left(\frac{14.46 \times 100}{14.55}\right)$.

The cell volume of 40.00 c.c. becomes 98.5 per cent $\left(\frac{40.00 \times 100}{40.61}\right)$.

Colour index = $\frac{\text{Percentage of hæmoglobin}}{\text{Percentage of red cells}} = \frac{99.4}{99.4} = 1.00$.

Volume index = $\frac{\text{Percentage of cell volume}}{\text{Percentage of red cells}} = \frac{98.5}{99.4} = 0.99$.

Saturation index = $\frac{\text{Percentage of hæmoglobin}}{\text{Percentage of cell volume}} = \frac{\text{Colour index}}{\text{Volume index}} = \frac{99.4}{98.5} = 1.01$.

SUMMARY.

1. Standards for the normal averages have been worked out by the study of bloods of 101 healthy young women from the Bombay Presidency between the ages of 16 and 30 years. The findings are given in the table below:—

Averages and range of variations in normal findings of 101 women.

	Mean.	Minimum.	Maximum.	Standard deviation.	Percentage of subjects within significant variation.
Red cells, million per c.mm.	4.47	3.50	5.35	± 0.330	73
Hæmoglobin, grammes per 100 c.c.	12.99	10.46	16.01	± 1.10	67
Cell volume, c.c. per 100 c.c.*	36.27	28.40	44.67	± 3.050	70
Hæmoglobin coefficient, grammes per 100 c.c.	14.55	11.40	16.40	± 0.960	71
Volume coefficient, c.c. per 100 c.c.	40.61	33.47	47.68	± 2.670	71
Colour index ..	1.00	0.78	1.13	± 0.065	71
Volume index ..	1.00	0.92	1.17	± 0.065	75
Saturation index ..	1.00	0.83	1.19	± 0.068	72

* The cell volume given in the table is the average of the actual volumes determined in oxalated blood without any correction being made for shrinkage due to the oxalate used.

2. Our average hæmoglobin value is based on determinations made with the van Slyke oxygen capacity method; and just as in the case of our average value for men it tallies with the only other figure for women determined by the same method. The significance of this observation is discussed and further work is suggested.

3. Our cell volume coefficient for women is very close to the value we obtained for men (40.83 c.c.) and shows that average size of the red cell in men and women is the same.

Our thanks are due to Dr. Jivraj Mehta, the Dean of the S. G. S. Medical College and K. E. M. Hospital, to Sir Mangaldas Mehta, Principal Medical Officer, Nowrosjee Wadia Maternity Hospital, and to Dr. J. Jhirad, Chief Medical Officer, Cama and Albless Hospitals, for giving us facilities for obtaining samples of blood, and to the students and nurses who volunteered.

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HÆMATOLOGICAL INVESTIGATIONS IN SOUTH INDIA.

Part I.

THE ESTIMATION OF HÆMOGLOBIN.

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THE present paper describes an investigation of the hæmoglobin content of the blood in healthy groups of young men and women in South India, which was undertaken for the purpose of establishing suitable standards of comparison. Investigations on hæmoglobin content have been carried out in Bombay by Sokhey (1929) and in Calcutta by Napier and Das Gupta (1935*a* and *b*) but hitherto no work on average levels has been done in South India. In undertaking work on the hæmoglobin content of blood, it is first of all necessary to consider the question of methods and the principles underlying them. This paper, the first of a series, is largely concerned with this problem, which is of great importance.

THE STATEMENT OF 'NORMAL' HÆMOGLOBIN VALUES.

It has been the custom to state the amount of hæmoglobin in the blood as such and such a percentage. The exact significance of the percentage recorded usually remains obscure in the absence of information as to the exact value of 100 per cent, which varies widely according to the method adopted. The following table (Kilduffe, 1931) gives hæmoglobin values which are taken

to be equivalent to 100 per cent in various commonly used methods of determination:—

Method.	Grammes of hæmoglobin per 100 c.c. of blood equivalent to '100 per cent'.
Dare .. before 1916 : 13·17 ; since 1916 ..	16·90
Haldane	13·80
Tallqvist	13·80
Oliver	15·00
Von Fleischer Meischer	15·80
Newcomer	16·92
Sahli	17·20

A variation of 13·17 to 17·20 grammes.

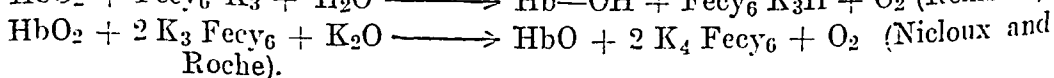
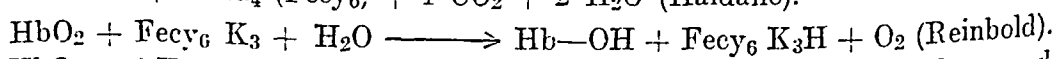
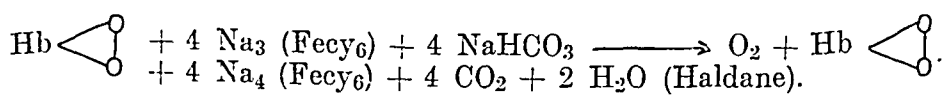
To avoid confusion, it is simpler to state the amount of hæmoglobin in blood in grammes per 100 c.c. This method of statement has been warmly advocated by several workers (Kilduffe, *loc. cit.* ; Osgood and Haskins, 1931 ; Todd and Sanford, 1927 ; Lindsay, Rice and Selinger, 1925-26 ; Napier and Das Gupta, 1935*a* and *b*).

PRINCIPLES AND METHODS OF HÆMOGLOBIN DETERMINATION.

(1) *The iron content of the blood.*—Wong (1928) has described a method of calculating the amount of hæmoglobin from the amount of iron contained in the blood. He finds a definite proportion between iron and hæmoglobin. Sobel and Dreker (1933), using Wong's technique slightly modified, conclude that there is no constant relation between hæmoglobin and iron concentrations in blood. There is also evidence that iron is present in blood outside the red blood cells.

(2) *The spectrophotometric method.*—The absorption band of crystalline hæmoglobin or oxyhæmoglobin can be studied. Williamson (1916) investigated the hæmoglobin values of 919 normal individuals in Chicago by a spectrophotometric technique. He prepared crystalline human hæmoglobin, and this preparation was used for a standard in his extensive determinations. It is not, however, yet certain that human hæmoglobin can be obtained absolutely free from impurities. Further, there is evidence that the globin part of the Hb molecule shows different absorption bands in infants and in adults.

(3) *The oxygen-capacity method.*—The most important property of hæmoglobin is its ability to transport oxygen from the lungs to the tissues, and a method based on this property is rationally to be preferred to other methods less closely related to physiological function. One molecule of oxygen is liberated from one molecule of oxyhæmoglobin, the following formulæ for the reaction having been put forward:—



METHOD USED IN THE PRESENT INVESTIGATION.

The van Slyke-Neill technique was employed to determine the oxygen capacity of blood samples which were used as the standard of comparison. The method described by van Slyke and Neill (1924) was exactly followed. Having ascertained in this manner the oxygen capacity of a sample of blood, from which the hæmoglobin content can be calculated by applying Hüfner's factor, we have in Wu's (1923) colorimetric method, as modified by Bing and Baker (1931), an accurate and simple technique for estimating Hb in terms of the standard. The results reported in this paper were all obtained by using Bing and Baker's technique slightly modified. The advantages of this method, as compared with others in common use, will be discussed later.

BING AND BAKER'S MODIFICATION OF WU'S COLORIMETRIC METHOD.

The method is based on the pseudoperoxidase reaction which is due entirely to hæmoglobin. The reagents necessary are as follows:—

(a) *Benzidine*.—The purification of benzidine for the test has been discussed by Bing (1932). In our work we proceeded as follows:—

About 20 grammes of benzidine are dissolved in 200 c.c. of ethyl alcohol by heating and the solution filtered. About 1 g. of Norite charcoal is added to the filtrate, which is stirred and maintained at a temperature of $50^{\circ}\text{C.} + 15^{\circ}\text{C.}$ over an electric heater for 15 to 20 minutes. The warm solution is filtered and the charcoal washed with about 10 c.c. of alcohol. In our experience the addition of charcoal and the heating, filtering, and washing with alcohol often had to be repeated 10 to 12 times in order to obtain a practically colourless filtrate. To the filtrate distilled water is added to bring the alcohol solution approximately to 50 per cent by volume. The solution is now kept in cold storage for a day to allow the benzidine to crystallize. The moist benzidine crystals in alcohol are then filtered under suction in a Buchner funnel, washed with cold 50 per cent alcohol until thoroughly dry, and then stored in a brown bottle away from light.

This elaborate purification has been advocated by Bing and Baker because blank experiments with unpurified benzidine give a distinct colour although no blood is present. McFarlane and Hamilton (1932), however, point out that there is no necessity for such an elaborate purification of benzidine, and maintain that Hb estimations of equal accuracy can be obtained by using Merck's 'Benzidine Suitable for Detecting Blood' without any purification treatment. There may perhaps be no great disadvantage in using a blank solution giving a certain amount of colour since, for determinations in blood, fixed amounts of the reagents are used for both standard and unknown. In our opinion Merck's 'Benzidine Suitable for Detecting Blood' may legitimately be used.

The stock solution of benzidine is then prepared by dissolving 1.0 g. of the purified benzidine crystals in 20 c.c. of glacial acetic acid. Thirty c.c. of distilled water and 50 c.c. of absolute alcohol are then added, and the solution well mixed and stored in a brown stoppered bottle away from light. The 1 per cent benzidine reagent referred to in our hæmoglobin investigations was prepared in this manner.

(b) *Twenty per cent acetic acid*.—Glacial acetic acid diluted 1 in 5 with distilled water.

(c) *Hydrogen peroxide*.—One volume of 'Merckozone' is diluted 4 times to give a 2 per cent solution of hydrogen peroxide. This was found in our investigations

to give a good and stable colour with the benzidine-blood mixture. Bing and Baker (*loc. cit.*) originally suggested a 0.60 per cent of solution of H_2O_2 . McFarlane and Hamilton (*loc. cit.*) have shown that 1.5 to 2.0 per cent H_2O_2 is the optimum strength for producing stable and lasting colour.

(d) *The preparation of a standard blood solution.*—A sample of blood is investigated for its oxygen capacity by the van Slyke-Neill apparatus and its Hb content calculated. This being known, a suitable standard, convenient for comparison with the blood samples under investigation, can be prepared. The following was used in our investigations. Twenty c.mm. of blood (measured 4 times from a 5 c.mm. Gower's hæmoglobinometer pipette) are laked in 40 c.c. of a 1 per cent solution of boric acid. The walls of the pipette are thoroughly washed by repeated sucking in and blowing out.

Technique of test.—The finger tip is pricked with a sharp pin and blood sucked into a dry 5 c.mm. Gower's pipette past the 5 c.mm. mark. The column of blood is lowered exactly to the mark and the tip of the pipette wiped clear of blood with a small piece of blotting paper. The blood is now blown into 10 c.c. of distilled water in a test-tube. Blood sticking to the walls of the pipette is washed into the water by the repeated sucking in and blowing out of water. The test-tube is shaken to lake the blood uniformly, and after a few minutes, when the laked blood is homogeneously mixed, 0.5 c.c. of the blood solution (measured in a clean 1 c.c. graduated pipette) is added to 1.0 c.c. of the 1 per cent benzidine reagent standing in a clean test-tube of about 15 c.c. to 20 c.c. capacity. Then exactly 0.5 c.c. of 2 per cent H_2O_2 is added. These quantities are half those used by Bing and Baker.

To another test-tube containing 1.0 c.c. of benzidine reagent and marked 'standard', 0.5 c.c. of stock standard blood solution is added, and the subsequent procedures take place in the same way.

The tubes are shaken and placed in a rack to develop colour. The colour immediately becomes green, then changes to blue, and finally to dark purple. The deepest colour is obtained in from 1 to $1\frac{1}{2}$ hours after which it remains constant for at least 12 hours (McFarlane and Hamilton, *loc. cit.*). An hour to $1\frac{1}{2}$ hours after the addition of H_2O_2 , 10 c.c. of 20 per cent acetic acid are added to each of the test-tubes. These are shaken to ensure thorough mixing and about 10 minutes later colour comparisons of the standard and the test solutions are made in a colorimeter (we used a Klett-Bio colorimeter), the standard being set at 10.

Results are calculated as follows:—

$\frac{10}{R} \times S = \text{grammes Hb per cent}$, S being the number of grammes of Hb in the standard solution and R the colorimeter reading for the test solution.

THE INVESTIGATION OF HÆMOGLOBIN LEVELS IN SOUTH INDIANS.

A group of 125 students and young doctors in Madras (males) and a group of 62 women students living in a residential college in the same city were investigated. The ages of the male subjects lay between 18 and 25 (a few were above 25), while that of the female subjects lay between 17 and 22. All were apparently in good health, and there was no reason to suppose that any of them was suffering, or had

recently suffered, from malaria or helminthic infections. A blood film was taken in each case and differential counts made with a view to excluding the latter. The results of the differential counts will be reported in a later communication.

The investigations were carried out by the technique described in the previous sections, the Hb value of the test samples being determined by comparison with a standard blood solution of known oxygen capacity, using Bing and Baker's method.

The results of these investigations are given in Tables I and II, the frequency curves of the hæmoglobin values being shown in the Graph:—

TABLE I.

Hæmoglobin values of 125 healthy young men (Madras).

Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.
1	14.20	26	18.46	51	16.72	76	12.65	101	12.25
2	15.45	27	19.96	52	17.30	77	14.90	102	13.52
3	16.30	28	18.00	53	16.86	78	16.20	103	13.52
4	15.25	29	18.90	54	17.48	79	17.60	104	12.38
5	14.25	30	17.30	55	17.48	80	13.12	105	17.50
6	17.85	31	14.10	56	16.48	81	13.33	106	18.25
7	17.70	32	13.20	57	17.30	82	16.72	107	17.15
8	14.60	33	14.10	58	17.60	83	15.56	108	17.73
9	17.50	34	12.24	59	20.28	84	12.90	109	16.32
10	17.20	35	19.30	60	19.70	85	14.46	110	13.42
11	17.85	36	17.75	61	20.01	86	16.48	111	14.53
12	14.05	37	17.46	62	19.70	87	16.32	112	15.92
13	19.40	38	16.60	63	20.28	88	17.90	113	14.33
14	16.82	39	16.86	64	18.40	89	16.32	114	16.88
15	17.25	40	16.86	65	19.10	90	16.32	115	15.56
16	14.10	41	16.86	66	17.75	91	17.48	116	15.12
17	14.10	42	18.10	67	16.82	92	15.25	117	15.25
18	14.85	43	17.72	68	19.48	93	16.48	118	14.53
19	16.10	44	19.40	69	18.90	94	18.75	119	12.45
20	14.97	45	17.90	70	18.25	95	13.32	120	14.80
21	16.70	46	18.40	71	18.25	96	14.05	121	19.65
22	19.55	47	20.28	72	15.46	97	16.20	122	15.25
23	19.96	48	17.60	73	16.86	98	20.28	123	14.45
24	18.36	49	18.92	74	17.48	99	14.12	124	16.72
25	19.95	50	16.20	75	14.54	100	13.12	125	20.00

(i) Mean hæmoglobin value = 16.57 grammes per 100 c.c.

(ii) Standard deviation = 2.08.

(iii) Coefficient of variation = 12.50 per cent.

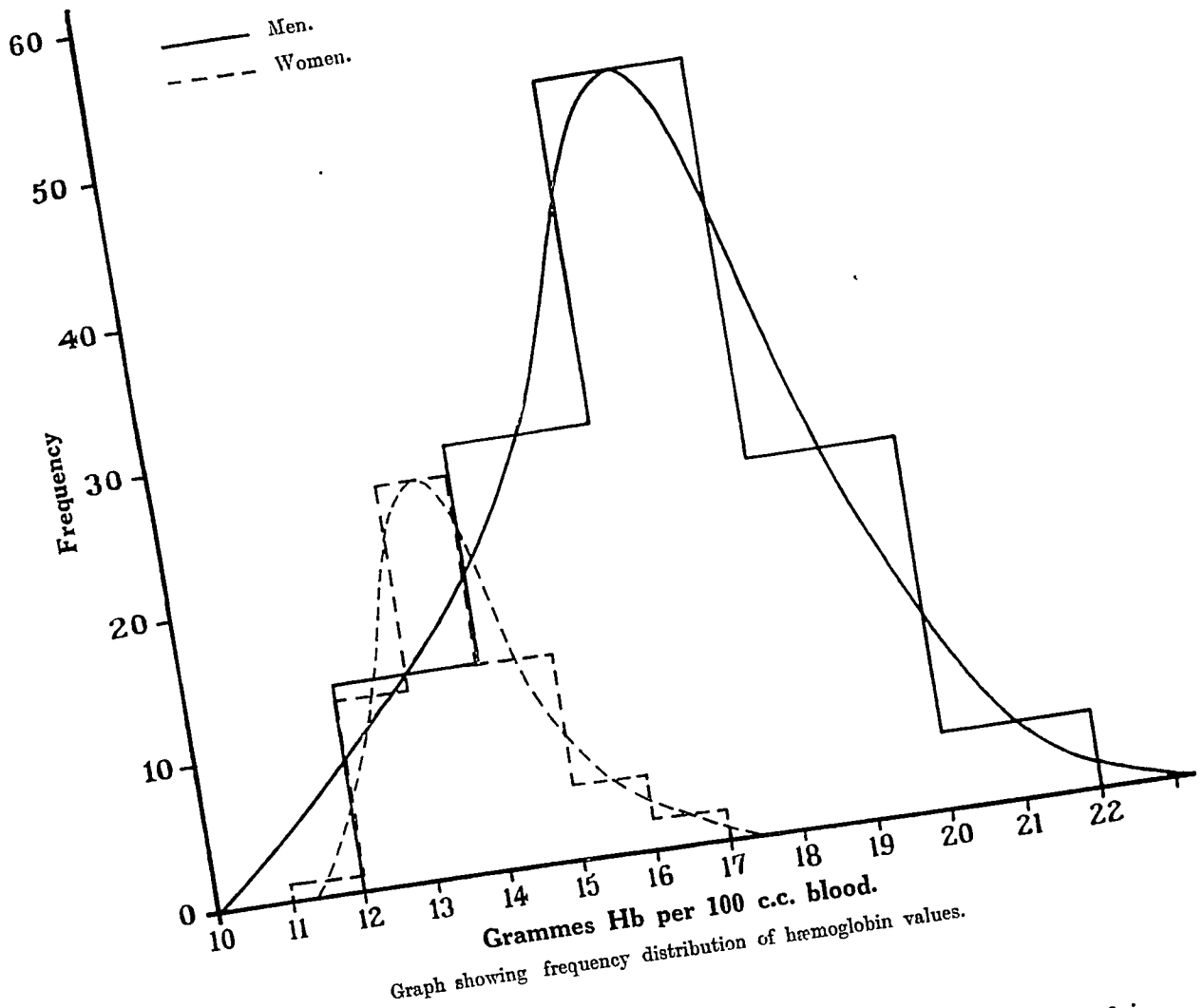
TABLE II.

Hæmoglobin values of 62 healthy young women (Madras).

Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.	Serial number.	Hb, g. per 100 c.c. blood.
1	14.08	17	12.92	33	13.10	49	15.16
2	15.08	18	14.42	34	15.08	50	13.98
3	13.04	19	12.60	35	13.16	51	13.16
4	13.10	20	12.88	36	13.16	52	16.26
5	13.52	21	12.56	37	12.56	53	15.16
6	14.02	22	13.22	38	12.72	54	14.60
7	14.22	23	13.22	39	12.88	55	14.70
8	13.76	24	13.46	40	15.08	56	13.60
9	13.82	25	13.22	41	14.22	57	16.12
10	13.64	26	14.08	42	13.64	58	13.98
11	12.72	27	14.42	43	13.22	59	12.58
12	12.94	28	13.82	44	13.46	60	12.50
13	11.82	29	13.16	45	14.16	61	14.60
14	12.72	30	14.22	46	13.82	62	14.60
15	13.28	31	13.46	47	14.56
16	13.40	32	13.82	48	12.94

- (i) Mean hæmoglobin value = 13.73 grammes per 100 c.c.
(ii) Standard deviation = 0.93.
(iii) Coefficient of variation = 6.70 per cent.

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DISCUSSION.

In considering the value of the technique of haemoglobin estimation used in our experiments, certain defects inherent in methods in current use may be pointed out. Commonly used methods include :—

- (1) The Tallqvist scale. A drop of blood is absorbed on blotting paper and the colour matched against shades of red representing percentage Hb values.

- (2) Haldane's method, in which CO gas is passed into laked blood to convert the Hb into CO-Hb, and the colour developed is compared with a standard solution in a sealed tube. The standard solution is said to remain unchanged indefinitely.
- (3) Methods in which acid hæmatin is used as standard, e.g., Sahli's, Hellige's, etc. The unknown blood samples are converted into acid hæmatin by the addition of HCl and matched against the standard hæmatin solution contained in a sealed tube.
- (4) Coloured glass or coloured plates are used as standards, comparisons being made with the naked eye. Examples: Dare's and Haden's methods.
- (5) The unknowns are compared with coloured glass or coloured plates in a colorimeter. Examples: Cohen and Smith's (1919) method; Newcomer's method.

The 'Tallqvist' method is generally recognized to be inaccurate, although widely used. At best it gives only an approximation to the true Hb figure. Lindsay *et al.* (*loc. cit.*) remark that it is rare to find a Dare hæmoglobinometer giving sufficiently accurate results unless re-standardized from time to time, a procedure not often followed in clinical practice. In the Sahli and Hellige's methods, an acid hæmatin standard is used. Haden (1930-31) says that in time the acid hæmatin suspension settles and the colour fades.

In Newcomer's (1923) method the standard is a 'high transmission yellow signal glass' which is compared with the test sample, diluted with 1 per cent HCl, in a colorimeter. In spite of the additional accuracy obtained by the use of a colorimeter, it appears that the method is open to criticism. According to Osgood and Haskins (*loc. cit.*), Newcomer's method may give an error as high as 13 per cent. Senty (1923) comes to the conclusion that in 40 per cent of Hb estimations carried out by Newcomer's method there is a 10 per cent error.

Cohen and Smith's method, in which acid hæmatin is used as a standard, represents an improvement over Sahli's method in that a colorimeter is used. They claim that a suitably prepared acid hæmatin standard remains unchanged for at least three months.

Sources of error in current methods of Hb determination include:—

- (a) Inexact calibration of the pipettes.
- (b) Deterioration of the permanent standard.
- (c) Differences in the character, depth and thickness of glass tubes used to contain standard and test solution (Sahli).
- (d) The matching of colours by the naked eye.

In Dare's hæmoglobinometer blood is run into a groove between two glass plates. Very slight variations in the depth of the small column of blood may give rise to errors of considerable magnitude.

A general criticism of all current methods of Hb estimation has been made by Osgood and Haskins, who maintain that 'most hæmoglobin figures in the literature are valueless, either because a grossly inaccurate method has been used or because

the method or the figure for the number of grammes of hæmoglobin corresponding to 100 per cent or the particular instrument is not given'.

Methods which are of use as a rough guide in clinical medicine are unsuitable for scientific investigation.

THE ADVANTAGES OF THE METHOD EMPLOYED IN THE PRESENT INVESTIGATION.

(1) The small quantity of blood (5 mm.) required can be very accurately measured in a suitable pipette (Gower's).

(2) The colour developed with the benzidine reagent is deep compared with that of diluted blood. Deeply coloured solutions are more easy to match than lightly coloured ones.

(3) The colour comparisons are made in a colorimeter.

(4) A larger number of estimations can be carried out within a day by this method than by any other.

(5) The reagents necessary are easily obtainable. (Merck's 'Benzidine Suitable for Detecting Blood' can be used instead of benzidine prepared in the laboratory.) The chemical manipulations are all of a very simple nature.

(6) The stock standard blood solution, the oxygen capacity of which has been determined, will remain without deterioration for at least 15 days. Before deterioration has taken place, the Hb value of a fresh sample of capillary blood can be determined colorimetrically in terms of the original standard; this sample then becomes the standard. In this way the standard can be 'kept alive' for a considerable period. It is, however, advisable to make a fresh standard from time to time using the gasometric technique. The method is suitable for use in group hæmoglobin determinations and in large hospitals to which physiological laboratories are attached. The employment of the van Slyke gasometric apparatus to determine the oxygen capacity of the standard blood solution is an essential part of the technique.

As a further improvement the use of a potentiometer, a galvanometer, and a photo-electric cell to compare the intensity of the colour of standard and test solutions is suggested. The potentials for different intensities of colour could be determined from a series of blood samples of known oxygen capacity, and recorded graphically; the Hb value of test solutions could then be determined by comparing the potentials with those given in the Graph.

In most methods of Hb determination, the standard is based on venous blood, while the test samples consist of capillary blood. Price-Jones, Vaughan and Goddard (1935) found no difference between the Hb content of venous and capillary blood in a series of European subjects. Napier and Das Gupta (*loc. cit.*), however, observed that in Indians the Hb content of the capillary blood was 1.0 gramme to 1.4 grammes higher than that of venous blood.

HÆMOGLOBIN VALUES OBTAINED IN THE PRESENT INVESTIGATION.

In the male groups the average Hb value was 16.57 g. per 100 c.c. of blood, with a standard deviation of 2.08 and a coefficient of variation of 12.50 per cent.

The variation in this group was greater than in the female group, probably because the young women investigated were all living in the same institution. Sokhey (*loc. cit.*), using the van Slyke technique, carried out Hb determinations on 121 healthy young Indian men in Bombay. He found the mean to be 15·36 g. per cent.

In the female group in Madras, the mean Hb was 13·73 g. per 100 c.c., with a standard deviation of 0·93 and a coefficient of variation of 6·7 per cent. Sokhey found the average Hb to be 12·99 g. in 101 women in Bombay. Our mean figures are in fairly close agreement with those of Sokhey.

Napier and Das Gupta (1935*b*) have recently investigated the Hb content of the blood of 50 healthy Indian men in Calcutta and found the mean to be 14·77 g. per cent.

Our results support the conclusion of these workers that 'there is little evidence for the statement that often finds its way into textbooks, namely that the hæmoglobin percentage in the blood of Indians is usually lower than in that of Europeans'.

It is desirable, in describing a series of Hb determinations carried out in large groups, to give, in addition to the mean value, the standard deviation and the coefficient of variation.

SUMMARY.

(1) The advantages of expressing hæmoglobin values in grammes per 100 c.c. of blood rather than as percentages are emphasized. It is pointed out that in describing the 'normal' a range should be given.

(2) A technique, involving the determination of oxygen capacity by van Slyke's apparatus, and the use of Bing and Baker's modification of Wu's method to compare test samples with a standard sample of known oxygen capacity, is described. The merits of the method followed, as compared with those of other methods in current use, are pointed out.

(3) In a group of 125 healthy young South Indian males the average hæmoglobin value was 16·57 grammes per 100 c.c. of blood, with a standard deviation of 2·08 and a coefficient of variation of 12·5 per cent. In a group of 62 healthy young South Indian women the corresponding figures were 13·73 grammes, 0·93 (σ) and 6·7 per cent (C.V.) respectively. As far as the present investigation goes, there is no ground for supposing that Hb values are lower in healthy Indians than in healthy Europeans.

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HÆMATOLOGICAL INVESTIGATIONS IN SOUTH INDIA.

Part II.

THE EFFECT OF THE ADMINISTRATION OF IRON ON THE HÆMOGLOBIN LEVEL IN INDIAN GIRLS AND YOUNG WOMEN.

BY

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IN the previous paper (Sankaran and Rajagopal, 1938) mean hæmoglobin values of groups of healthy young men and women in South India were reported. The values were 16.57 g. and 13.73 g. per cent respectively. The question arose whether these figures represent 'normal' or 'optimum' levels, and to clarify this point further an investigation of the effect of iron administration on hæmoglobin was undertaken.

EXPERIMENTAL.

Two residential institutions for girls and young women were chosen as the venue of the investigation. One of these was a Mission school situated on the outskirts of a small town in a rural area; the other was a residential college in Madras, in which research on mean hæmoglobin levels had previously been carried out. These institutions will be called 'Mission school' and 'college' respectively throughout the paper.

In the Mission school experimental and control groups were constituted as follows: Twenty-four girls were given iron and 24 acted as controls, the lowest age being 10 and the highest 16 years. Experimental and control groups were further subdivided into two sub-groups of 12, one of which was composed of girls who had not menstruated, the other of girls who had attained puberty; this subdivision was made because it was thought that menstruation might have some influence on Hb levels. No other selective criterion was applied beyond the arrangement of the groups to obtain rough comparability with regard to age and the attainment of puberty or otherwise.

The girls were of South Indian Tamil stock. All appeared healthy, and there was no history or evidence of recent serious illness. Anti-helminthic treatment, the chief purpose of which was the eradication of hookworm, was given as a routine to all children entering the school and repeated at periodic intervals. Differential leucocyte counts were made by us in each case, and provided no evidence of helminthic infection or malaria. The diet of the girls, based on milled rice, was a fairly well-balanced one as judged by the standards obtaining in South India. The daily intake of total iron per consumption unit was estimated at about 23 mg.

In the women's college two similar groups of 24, one to receive iron, the other to act as control, were selected for the experiment. Their ages ranged from 16 to 22, and all had passed the age of puberty. Most of the students were of Tamil or Malayalee stock with a sprinkling of Kanarese and Telugu. Many belonged to relatively prosperous families. Blood examinations provided no evidence of helminthic infection or malaria. General health was good.

The diet consumed was based on milled rice but contained other foods, including vegetables in fair quantities. Total daily intake of iron per consumption unit was calculated to be about 25 mg. to 30 mg. This estimate takes no account of losses in cooking or of the 'availability' of the iron contained in the diet.

THE ADMINISTRATION OF IRON.

Six grains* of ferrous sulphate, equivalent to about 120 mg. of iron (Witts, 1936), in sealed gelatine capsules, were given daily for four weeks (3-grain capsules twice daily) to the 24 girls in the Mission school, i.e., each girl received 168 grains in all. In the college a similar dose of ferrous sulphate was given in the same way for six weeks, a total of 252 grains for each young woman. The administration of each dose was carefully supervised and recorded, and a watch was kept for the occurrence of such symptoms as nausea, loss of appetite, vomiting, gastric pain, etc. Three of the 48 subjects showed mild nausea, and in these cases the capsules were replaced by a mixture containing 30 grains of ferri et ammon citras, to be taken twice daily.

The Hb estimations were made by Bing and Baker's (1931) modification of Wu's (1923) method, as described in Part I of this series, and expressed as grammes per cent by comparison with a standard blood solution of known oxygen capacity. Blood was always taken at the same time of day.

RESULTS.

Tables I and II give the Hb values determined at periodic intervals in the two institutions. The same results are displayed graphically in the Chart. In Table III the levels obtained in the experimental and control groups are compared and the results of statistical analysis given. The criterion of significance was the conventional one of 'more than twice the standard error of the difference between the two means' the formula $\sqrt{\frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}}$ being applied, when σ = the standard deviation and n_1 and n_2 the number in each of the two groups.

* 1 grain = 64.8 mg.

TABLE I.
Hæmoglobin levels (grammes per 100 c.c.) of groups in the Mission school receiving and not receiving iron.

EXPERIMENTAL GROUP RECEIVING IRON.					CONTROL GROUP (NO IRON).				
Commencement of the investigation.	2 weeks later.	4 weeks later.	2 months after iron administration was stopped.		Commencement of the investigation.	2 weeks later.	4 weeks later.	2 months after iron administration was stopped.	
A.	17.34	17.74	15.16	M. G. R.	15.06	13.68	16.34	10.60	
S. A.	15.54	17.56	15.22	C.	16.58	15.76	16.16	15.08	
K. A.	14.86	15.26	13.96	R. S.	16.30	16.62	16.00	13.10	
P. U. S.	14.86	19.12	15.30	S.	14.22	14.46	13.48	9.80	
T. T.	18.86	19.54	19.10	P.	14.70	16.41	16.41	15.22	
N. G.	17.24	15.96	15.16	N. S.	14.92	13.24	14.70	13.52	
M.	15.00	17.66	16.40	S. W.	18.40	13.50	13.82	15.96	
N.	19.74	21.46	18.10	S. A.	16.40	13.28	20.02	18.00	
M. T.	16.94	16.62	16.22	S. E.	15.00	13.98	16.74	15.00	
S. P.	15.22	17.00	16.40	T.	17.54	14.72	17.26	15.38	
T. D. N.	17.44	19.54	17.24	P. A.	17.86	14.58	16.44	15.62	
S. V.	15.51	13.88	17.64	K. S.	12.04	13.72	15.80	12.76	
L. D.	15.46	15.76	16.94	K. R.	14.64	..	12.44	9.74	
S. D.	18.64	18.32	17.34	C. V.	14.20	15.50	15.70	15.08	
P. M.	17.64	17.24	17.34	R. P.	16.48	13.62	16.34	16.66	
J. J.	15.88	15.38	17.24	K. G.	15.08	13.72	15.62	15.52	
N. R.	15.08	15.98	13.40	D. S.	11.72	13.50	15.80	12.56	
C. S. M.	16.66	17.66	15.64	P. J.	17.86	16.96	16.16	17.44	
R. M.	15.08	17.78	17.34	G.	16.76	13.78	14.60	12.66	
A. A.	15.80	16.34	14.50	S. J.	12.82	17.72	12.98	12.88	
R. A.	19.10	20.38	17.14	R. A.	13.56	14.38	13.56	13.10	
A. D.	14.92	17.66	16.12	R. A. S.	13.52	14.46	12.98	14.28	
S. G.	16.58	16.26	14.64	R. S.	17.76	17.66	14.94	16.58	
D. G.	14.16	15.30	16.04						
Mean Hb. =	16.35 g.	17.23 g.	16.21 g.	Mean Hb. =	15.39 g.	14.75 g.	15.23 g.	14.23 g.	
σ =	1.57	1.58	1.41	σ =	1.83	1.40	1.72	2.14	
C. V. =	9.6 per cent.	9.4 per cent.	8.7 per cent.	C. V. =	11.8 per cent.	9.5 per cent.	11.3 per cent.	15.0 per cent.	

TABLE II.

Hæmoglobin levels (grammes per 100 c.c.) of groups in the college receiving and not receiving iron.

EXPERIMENTAL GROUPS RECEIVING IRON.					CONTROL GROUP (NO IRON).				
Commencement of the investigation.	2 weeks later.	4 weeks later.	6 weeks later.		Commencement of the investigation.	2 weeks later.	4 weeks later.	6 weeks later.	
U. M.	13.22	16.94	17.54		F. R.	14.08	14.50	14.86	14.92
J. L.	14.08	16.94	17.54		D. L.	15.08	15.88	15.46	15.54
G. L.	14.42	17.76	17.86		P. M. M.	13.04	13.22	14.78	14.92
U. K.	13.82	16.94	19.10		N. S.	13.10	13.34	14.56	14.64
D. M.	13.16	15.70	15.62		C. G.	13.52	13.58	14.28	14.86
D. L. P.	14.22	17.54	17.62		V. J.	14.02	13.96	15.16	15.16
M. L.	13.46	16.78	17.76		S. M.	14.22	14.50	15.70	15.80
S. V. C.	13.82	17.14	18.18		A. G. V.	13.76	13.70	15.22	15.08
D. G.	13.10	16.76	17.54		S. M.	13.82	13.52	15.22	15.00
P. N.	15.08	20.00	19.86		N.	13.64	13.64	15.22	15.16
M. M. B.	13.16	17.42	18.98		D.	12.72	12.56	14.36	14.36
T. A. K.	12.56	18.64	19.74		S. L.	12.94	12.60	13.76	14.22
J. A. P.	12.72	17.44	18.64		J. S. M.	11.82	12.40	13.04	13.40
P. C.	12.88	16.94	18.18		S. A.	12.72	13.58	13.28	13.22
G. M. P.	15.08	18.86	18.64		C. A.	13.28	13.58	13.88	14.16
C. S. K.	14.22	17.54	19.22		T. M.	13.40	13.04	14.28	14.16
P. L.	13.64	16.66	17.54		P. L.	12.92	13.28	14.28	14.28
K. G.	13.22	19.36	19.60		G. M. M.	14.42	13.70	14.28	14.28
P. S.	13.46	16.48	18.64		O. S. T.	12.60	13.04	13.52	13.64
K. T.	14.16	18.08	22.00		T. S.	12.88	12.50	13.46	13.64
B. S. T.	13.82	18.08	19.36		K. P.	12.56	12.40	13.22	13.88
G. A.	14.56	16.48	17.76		C. R.	13.22	12.94	14.22	14.16
F. M.	12.94	17.54	20.00		C. M.	13.22	13.58	13.70	13.76
		16.48	17.44		K. E.	13.46	12.98	13.64	13.70
Mean Hb. =	13.69 g.	17.33 g.	18.44 g.		Mean Hb. =	13.31 g.	13.35 g.	14.25 g.	14.40 g.
σ =	0.74	1.00	1.25		σ =	0.71	0.78	0.78	0.66
C. V. =	5.4 per cent.	5.8 per cent.	6.8 per cent.		C. V. =	5.3 per cent.	5.8 per cent.	5.5 per cent.	4.6 per cent.

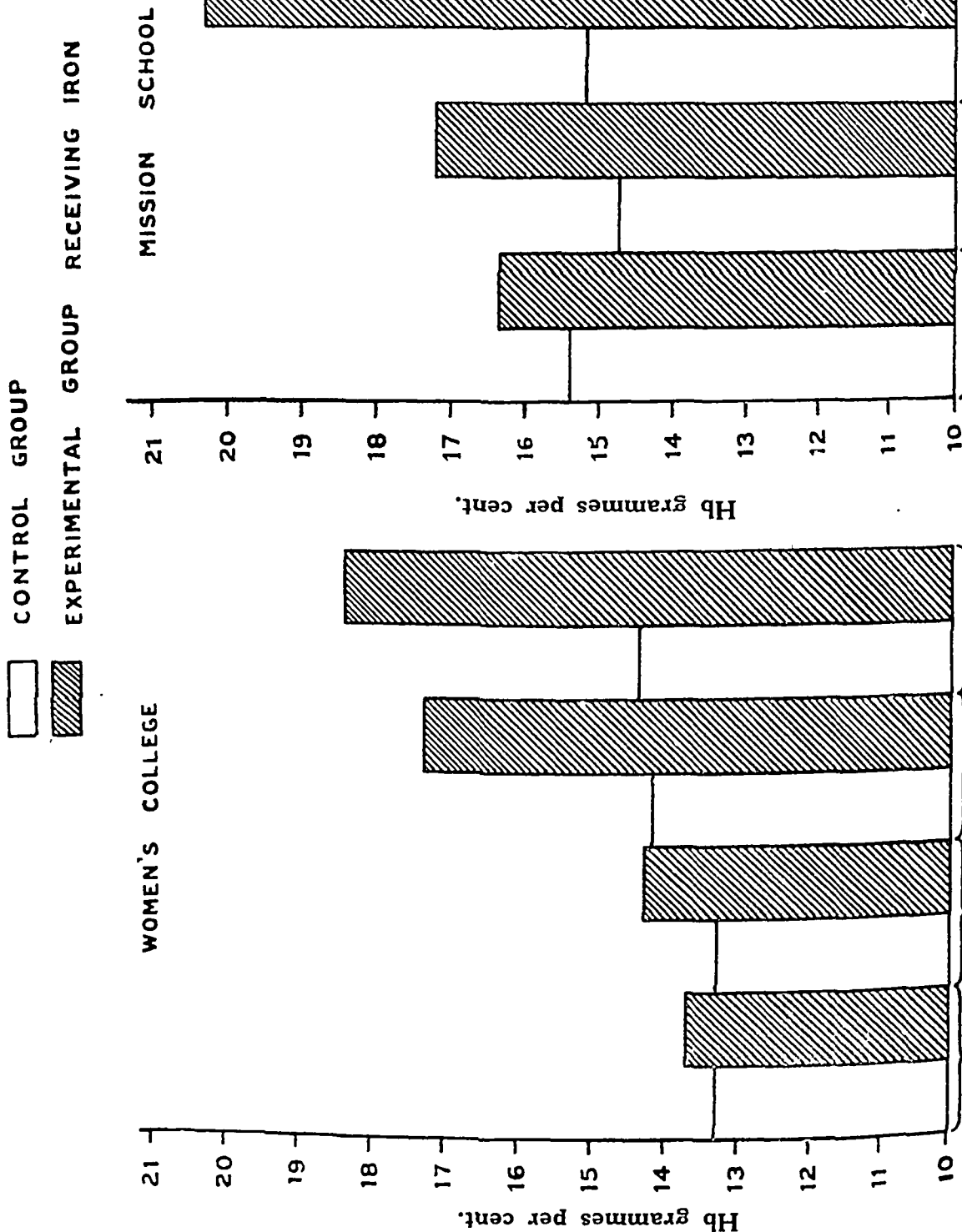
TABLE III.
The differences in the haemoglobin levels in the experimental and control groups with the results of statistical analysis.

(Hb figures = g. per cent.)

Commencement of the investigation.	2 weeks later.	4 weeks later.	6 weeks later.	2 months after iron administration was stopped.
MISSION SCHOOL:				
Mean Hb in experimental group.	16.35 g.			
Mean Hb in control group	15.39 g.	20.34 g.		16.21 g.
Excess Hb in experimental group.	0.96 g.	5.11 g.		1.98 g.
	Difference 'not significant'.	Difference 'significant'.		Difference 'significant'.
	17.23 g.	15.23 g.		14.23 g.
	14.75 g.			
	2.48 g.			
COLLEGE:				
Mean Hb in experimental group.	13.69 g.	17.33 g.	18.44 g.	
Mean Hb in control group	13.31 g.	14.25 g.	14.40 g.	
Excess Hb in experimental group.	0.38 g.	3.08 g.	4.04 g.	
	Difference 'not significant'.	Difference 'significant'.	Difference 'significant'.	
	14.31 g.			
	13.35 g.			
	0.96 g.			

'Significant' = the difference is greater than twice the standard error of the difference between the two means.

CHART.



The influence of iron on hemoglobin levels. The last column for the Mission school group indicates the level 2 months after the administration of iron.

It will be seen that in both institutions the rise in Hb following the administration of iron was statistically significant throughout the experiment. In Madras the mean level in the control group rose from 13.31 g. to 14.40 g. per cent during the experimental period. This increase is statistically significant and may perhaps be attributed to the greater interest in diet which naturally follows the presence of nutrition research workers in an institution: the diets consumed by all the pupils may have slightly improved and a larger intake of iron in food resulted. The rise in the control group was, however, much less than that observed in the group receiving iron.

In the Mission school hæmoglobin was determined two months after the giving of iron ceased. In the 'iron' group it had returned to the original level, but was significantly higher than in the controls.

MENSTRUATION AND HÆMOGLOBIN CONTENT.

There was no significant difference in the Mission school girls who had and had not attained puberty, and both groups showed roughly the same response to iron administration. In the college groups Hb levels were determined in 13 individuals during menstruation and in the intermenstrual period; the results indicate that Hb content is little influenced by menstruation.

DISCUSSION.

Widdowson and McCance (1936) have made a study of the effect of iron administration on the Hb level of 16 healthy English women between 20 and 30. The women were given ferrous sulphate or ferri et ammon citras for six weeks in amounts equivalent to 100 mg. of iron daily. The mean Hb level at the commencement of the experiment was 93 per cent on Haldane's scale (100 per cent = 13.8 g. per 100 c.c.). In eight subjects Hb increased by more than 10 per cent and in five cases the increase exceeded 15 per cent. Maximum levels were attained on the 3rd and 4th weeks. The same workers found that similar doses of iron had no effect on Hb levels in a group of 16 males, whose mean initial level was 104 per cent Haldane. Very little change was observed after two to five weeks' medication.

In Assam Napier and Das Gupta (1937) were able to increase the Hb of 'coolies' by 1.0 g. per cent in 10 days by administering 11 grains of ferrous sulphate daily. The initial Hb level was 11.2 g. per cent.

In the present investigation the original Hb levels were higher than those observed in the investigations of Widdowson and McCance and Napier and Das Gupta, the averages in the Mission school and college groups being 15.39 g. and 13.31 g. per cent respectively. We cannot account for the difference in the initial levels in the two institutions. In spite of the fact that these two levels were high, the effect of iron was to produce a marked increase in Hb content, and the level ultimately reached was considerably above those attained in previous experiments of this nature. In the Mission school a mean rise of 5.11 g. per cent took place in four weeks and in the college there was a rise of 4.04 g. per cent in six weeks. Widdowson and McCance remark that 'there is no proof that a hæmoglobin level

of 103 per cent is better for the women than a level of 93 per cent. It is possible that the lower level is the optimum, but the higher should probably be regarded as the physiological one'. Both levels—the level representing the mean in healthy women not receiving iron and the level attained after iron medication—were higher in the Indian subjects. Assuming that this difference is not due to variation in technique, the higher Indian figures may be due to racial or dietary factors. Further investigation of this problem is necessary.

Our investigations did not throw any further light on the problem raised by Widdowson and McCance—whether the optimum or physiological level is that present before or after iron medication. There was no obvious improvement in the health of the girls given iron, observed either by ourselves or their teachers. It might be argued, on physiological grounds, that a higher Hb content of the blood is advantageous in that it facilitates transport of oxygen and may lead to greater efficiency of tissue metabolism. Study of the basal metabolism in relation to Hb levels might produce interesting results.

It is interesting to observe that in the Mission school group 'the pre-iron' level was regained two months after the administration of iron ceased. To keep Hb at its 'maximum' level, continuous dosage with iron appears to be necessary.

The dose of ferrous sulphate usually recommended in secondary anæmia is 9 to 15 grains daily. In our investigation we chose a smaller dose, equivalent to 120 mg. iron daily, since the subjects were not suffering from anæmia. There is at present no information as to 'optimum' or minimum dosage of iron salts required to raise Hb in anæmic or apparently normal individuals. The capsules containing iron were taken easily by almost all the subjects, and mild gastric symptoms occurred in only three out of 48.

SUMMARY.

(1) The daily administration of ferrous sulphate to girls and young women living in institutions in South India produced a rise in the hæmoglobin content of the blood.

(2) In one institution the mean Hb initial level in the group receiving iron was 16.35 mg. per cent. After iron had been given for four weeks the mean level was 20.34 g. In another institution the levels before and after the administration of iron for six weeks were 13.69 g. and 18.44 g. per cent respectively. A rise was observed after two weeks of iron medication. Controls receiving no iron did not show similar increases.

(3) Hæmoglobin content was found to have returned to the original level two months after the administration of iron had been discontinued.

(4) No difference was found in Hb content of the blood of girls who had and had not attained puberty, or of girls during menstruation and in the intermenstrual period.

(5) It is pointed out that both the initial Hb levels observed in this investigation, and the levels attained after iron administration, are higher than those recorded by other workers.

(6) There is at present no information regarding 'optimum' Hb levels.

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THE ABSENCE OF EFFECTIVE IMMUNITY AFTER CURE OF PROTOZOAL INFECTIONS*.

BY

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INTRODUCTION.

THE study of immunity in recent years has led, more and more, towards the concept that the mechanisms involved are the same, in the main, whether the foreign material be virus, bacterium, protozoan, or even inanimate matter. While the mechanisms of resistance, then, may be admitted to be the same, or very similar, whatever the exciting cause, the results achieved by them would appear to vary, in degree if not in kind, according to whether the invading organism is a virus or bacterium on the one hand, or a protozoan on the other. The fact is brought out by experience that after viral and bacterial infections the immunity is often prolonged or even life-long, while after most protozoal infections it is evanescent or even non-existent. Thus diseases like yellow fever and enteric give prolonged immunity, while amœbic dysentery and malaria confer little or no immunity.

The apparent exceptions, both among viral or bacterial and among protozoal infections, may eventually prove susceptible of explanation and be shown to be no exceptions to the rule. Thus the transient immunity after the common cold and influenza among viral diseases and pneumonia, among bacterial diseases, and the long-standing or life-long immunity after oriental sore and kala-azar, among

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protozoal diseases, may be due in the former instances to complete disappearance of the infecting organism from the body and in the latter to continued persistence in the skin. These considerations would bring us to the concept of 'premunity' as defined by Sergeant *et al.* (1924), i.e., a condition of continued immunity due to the persistence of a latent infection which keeps continuously mobilized the protective forces of the organism.

While there is strong evidence in favour of this theory, so far as protozoal and viral infections are concerned, and the present study lends further support to it, the position with regard to bacterial infections is less clear and we do not propose to discuss it here.

OBJECTS OF THE PRESENT INVESTIGATION.

The usual course of events in a typical protozoal disease such as babesiasis or malaria is somewhat as follows:—

After infection there is an incubation period without symptoms. This is followed by a period of pyrexia, the appearance of large numbers of parasites in the peripheral circulation, and much blood destruction (acute attack). If the animal does not succumb the acute attack is followed by a stage in which there persists a latent or low-grade infection which prevents the animal being reinfected with the same strain of parasite. This is the condition of premunity.

It seemed to us that it would be of interest to determine whether the relative immunity present in this condition was entirely dependent on the low-grade infection and would disappear completely if true parasitic sterilization was effected.

MATERIALS AND METHODS.

For this inquiry we decided to employ infections with *Babesia canis*, *Plasmodium knowlesi*, and *Plasmodium cynomolgi*. The general plan of the experiments was the same in all infections. Animals were infected with a strain of each parasite and the infection was treated, if this was necessary to prevent the death of the animal. Such treated animals were only given sufficient treatment to keep them alive and allow them to develop sufficient immunity to establish a condition of low-grade infection.

Some of the animals were then further treated until they were not only clinically cured but considered to be definitely free of parasites. The two series, parasitically sterile and with low-grade infections, were then reinfected with homologous strains of parasites and the results noted.

In the first series of experiments with monkeys this procedure was carried out in full. In the case of the few experiments with *B. canis* and dogs the infection of dogs in a condition of premunity was not considered necessary as daily experience in the laboratory had already given sufficient evidence that such animals were usually refractory to fresh infections. In the second series of monkeys no 'premunity' animals were included in order to increase the number of animals available for the part of the experiment designed to show the absence or presence of immunity after parasitic sterilization.

EXPERIMENTS WITH *Babesia canis*.

For these experiments three dogs were selected as follows:—

- (a) A dog naturally infected.
- (b) A dog infected in the laboratory by infected ticks.
- (c) A dog infected in the laboratory by inoculation of infected blood.

These dogs were treated to obtain parasitic sterilization and, after an observation period during which daily examination of the blood revealed no parasites, the blood was inoculated into another susceptible dog to confirm the absence of parasites. Experience has shown the latter to be a most delicate test. If the test dog remained uninfected it was next proved not to be naturally resistant by placing on it infected ticks. In each case these produced infection after the usual incubation period of about four days.

The experimental dogs proved to be free of parasites by these tests were then exposed to infection to determine the absence or presence of immunity. The exposure to infection was brought about by

- (a) placing on them infected ticks, or
- (b) inoculating infected blood.

The details of three experiments are given below:—

EXPERIMENT I.

Dog 37—found naturally infected with B. canis.

6th March, 1935 to 22nd March, 1935 ..	Daily microscopical examination positive.
23rd March, 1935	Treated with 0.1 c.c. of Akiron R. (after previous treatment with trypan blue).
24th March, 1935 to 29th March, 1935 ..	Daily microscopical examination negative.
29th March, 1935	Blood inoculated into test pup 41 which remained uninfected (see below).
30th March, 1935 to 20th April, 1935 ..	Daily microscopical examination negative.
20th April, 1935	Infected adult ticks put on.
23rd April, 1935	Parasites appeared in blood.
24th April, 1935 to 29th April, 1935 ..	Progressive infection.
30th April, 1935	Dog died.

Test pup 41.

29th March, 1935	Inoculated with blood of cured dog 37.
30th March, 1935 to 13th April, 1935 ..	Daily microscopical examination negative.
13th April, 1935	Infected adult ticks (same series as used for dog 37) put on.
18th April, 1935	Parasites appeared in blood.

The test pup proved that dog 37 was uninfected at the time its blood was inoculated into the pup and the subsequent infection of the latter by ticks proved that it was not a refractory animal.

EXPERIMENT II.

Dog 43—infected in laboratory by ticks.

4th June, 1935	Infected adult ticks (same series as for dog 37) put on.
9th June, 1935	Parasites appeared in blood.
10th June, 1935 to 19th July, 1935 ..	Remained continuously infected.
19th July, 1935	Treated with 3.5 c.c. of 2 per cent trypan blue subcutaneously.

766 *Absence of Effective Immunity After Cure of Protozoal Infections.*

1st August, 1935 to 6th August, 1935	Daily microscopical examination negative.
6th August, 1935	Treated with 3.5 c.c. of 2 per cent trypan blue subcutaneously.
7th August, 1935 to 26th August, 1935	Daily microscopical examination negative.
26th August, 1935	Blood inoculated into test dog 57 which remained uninfected (<i>see below</i>).
27th August, 1935 to 5th September, 1935	Daily microscopical examination negative.
5th September, 1935	Infected adult ticks put on.
9th September, 1935	Parasites appeared in blood.
10th September, 1935 to 23rd September, 1935.	Progressive infection.
28th October, 1935	Dog died.

Test dog 57.

26th August, 1935	Inoculated with blood of cured dog 43.
27th August, 1935 to 16th September, 1935	Daily microscopical examination negative.
16th September, 1935	Infected ticks (nymphs and adults) put on.
19th September, 1935	Parasites appeared in blood.

The test dog proved the parasitic sterility of dog 43 and its own susceptibility to infection.

EXPERIMENT III.

Dog 52—infected in laboratory by inoculation of blood.

17th July, 1935	Inoculated with infected blood.
21st July, 1935	Parasites appeared in blood.
22nd July, 1935 to 5th September, 1935	Daily microscopical examination positive.
6th September, 1935	Treated with 4 c.c. of 2 per cent trypan blue subcutaneously.
7th September, 1935 to 13th September, 1935.	Daily microscopical examination negative.
13th September, 1935	Treated with 5 c.c. of 2 per cent trypan blue subcutaneously.
20th September, 1935 to 27th September, 1935.	Daily microscopical examination negative.
27th September, 1935	Blood inoculated into test pup 55 which remained uninfected (<i>see below</i>).
29th September, 1935	Infected blood inoculated.
1st October, 1935	Parasites appeared in blood.
2nd October, 1935 to 13th October, 1935	Progressive infection.
14th October, 1935	Dog died.

Test pup 55.

27th September, 1935	Inoculated with blood of cured dog 52.
28th September, 1935 to 14th October, 1935.	Daily microscopical examination negative.
15th October, 1935	Infected ticks (nymphs and adults) put on.
23rd October, 1935	Parasites appeared in blood.

This test pup proved the parasitic sterility of dog 52 and its own susceptibility to infection.

CONCLUSIONS DRAWN FROM THE *Babesia* EXPERIMENTS.

The experience of many workers, as well as our own experience, has shown that dogs with a chronic infection of *Babesia* cannot readily be superinfected. The present series of experiments appears to demonstrate that this immunity is completely lost as soon as a dog becomes completely free of parasites. So complete is the disappearance of immunity that there is not even the slightest lengthening

of the incubation period when the animal is exposed to infection by any method whatsoever.

FIRST SERIES OF EXPERIMENTS WITH MONKEY MALARIA.

Experiments on lines somewhat similar to those followed in the case of *B. canis* were conducted on monkeys infected with the Simian malarial strains—*Plasmodium cynomolgi* and *P. knowlesi*. In all the animals, infection was induced by the intraperitoneal injection of citrated blood drawn from a monkey in an acute or chronic stage of infection. Most of the animals employed belonged to the species *Sillemus sinicus* but some *S. rhesus* were also included. In both these species of monkey *P. cynomolgi* gives rise to a comparatively mild infection which, without any treatment, the acute stage of blood infection lasting on an average 11 days, is followed by the stage of chronic or latent infection. None of the monkeys infected with this strain of malaria was treated in the acute stage of the disease. *P. knowlesi* infection is, however, very severe in both the species of monkeys and particularly so in *S. rhesus*. In almost all cases, therefore, animals in this series were treated with quinine, given intramuscularly, during the acute primary attack and the relapses that followed. Only as few injections as were necessary to keep the animal from dying were given, with the object of giving free scope for the building up of natural resistance.

Some of the animals with a chronic or latent infection in either series were then taken up for treatment with the object of bringing about parasitic sterilization.

Anti-malarial drugs being notoriously defective in this respect, the following plan was arbitrarily adopted. The animals were given, on successive days, four intramuscular injections of 2 grains of quinine hydrochloride, followed by 3 grains of quinine sulphate by mouth on the two following days. After an interval of eight days, each animal was given on successive days, three intramuscular injections of atebirin musonate and, after a further interval of eight days, a second course of this drug was given on two successive days. The dose of atebirin musonate was 6 mg. per kilogram of body-weight.

The following three tests were applied as criteria of parasitic sterilization—absence of parasites in thick smears of blood made almost daily, the non-appearance of parasites in blood after provocative injections of calcium chloride intravenously and inability to infect a normal monkey when 2 c.c. of blood from the test monkey were inoculated into it intraperitoneally. It has been shown by Monteleone (1930) and Alberto Videla (1934) that, in the case of latent human malaria, intravenous injection of calcium chloride gives rise, on the second or third day, to the appearance of parasites in the blood, enlargement of the spleen and a positive Henry's flocculation test. In the treated monkeys injections of calcium chloride were commenced one month after the last dose of atebirin, three intravenous injections of 0.5 c.c. of 10 per cent calcium chloride being given on successive days. A week after the last injection 2 c.c. of blood were drawn from the vein of each monkey in citrated saline and injected intraperitoneally into a healthy susceptible monkey, *S. sinicus*. The animals reported as cured have stood these tests of parasitic sterilization.

The cured monkeys and monkeys with a latent infection were then given an intraperitoneal injection of 0.25 c.c. of fresh blood (citrated) drawn from a monkey infected with the homologous strain at the height of acute infection. At the same time one or two normal susceptible monkeys were also infected with the same dose. Thereafter, thick and thin smears were prepared daily from finger-blood and a numerical count of parasites was carried out by Sinton's method of counting with a standardized suspension of fowl's blood cells.

EXPERIMENT WITH *P. cynomolgi* INFECTION.

Eight animals are included in this experiment, namely four cured monkeys, three *S. sinicus* and one *S. rhesus*; three monkeys with a latent infection, two *S. sinicus* and one *S. rhesus*; and a normal *S. sinicus* as a control.

It has already been mentioned that this infection runs a mild course and does not kill the animals. A comparison of the infections in the different groups has, therefore, been sought by the number of parasites counted in one c.mm. of blood. If parasites were in excess of 900 per c.mm. of blood it has been called 'heavy infection'. The period of incubation, i.e., the number of days elapsing between the day of injection with infective blood and the appearance of parasites in the peripheral blood, and the period elapsing between the first appearance of parasites and the commencement of 'heavy infection' have been taken as criteria for judging the differences between the different groups of animals. Table I shows these differences:—

TABLE I.

Experiment with P. cynomolgi.

	Period of incubation (days).	The number of days elapsing between the first appearance of parasites and commencement of 'heavy infection'.	Duration of 'heavy infection' (days).
Normal monkey. C. 62 ..	3	1-2	16
Cured monkey. C. 5 ..	2	3	6
" " C. 9 ..	2	2	6
" " C. 15 ..	3	6	6
" " C. 30 ..	4	3	5
Monkey with latent infection. C. 14 ..	4	8	4
" " C. 18 ..	4	..	—
" " C. 43 ..	4	..	—

The term 'incubation period' cannot strictly be applied to animals with latent infection, but in all the three animals, parasites first appeared four days after superinfection, none having been seen during the four days.

The histories of the animals are given in greater detail below :—

(1) Normal monkey. C. 62 (*S. sinicus*).

18th October, 1935	Infected.
21st October, 1935	Parasites first appeared in blood.
22nd October, 1935 to 6th November, 1935			The animal had a 'heavy infection', the number of parasites per c.mm. being 2,000 on 22nd October, 1935, gradually increasing to 27,000 on 27th October, 1935, and then coming down slightly irregularly to 970 on 6th November, 1935. Thereafter the parasites were fewer in number and the animal lapsed into the stage of chronic infection.

(2) Cured monkey. C. 5 (*S. sinicus*).

18th October, 1935	Was infected after the previous latent infection had been cured.
20th October, 1935	Parasites first appeared.
23rd October, 1935 to 28th October, 1935			Had a 'heavy infection', the parasite count on successive days per c.mm. being 1,800, 2,700, 5,900, 6,100, 7,900, and 4,500. Later it passed into a chronic condition.

Previous history.—First infected 9th February, 1935. On 19th February, 1935, parasites first appeared. Was superinfected on 23rd May, 1935, but never developed a good infection. Treatment commenced on 8th July, 1935.

(3) Cured monkey. C. 9 (*S. sinicus*).

18th October, 1935	Was infected after the previous latent infection had been cured.
20th October, 1935	Parasites first appeared.
22nd October, 1935 to 27th October, 1935			Had a 'heavy infection', the parasite count on successive days per c.mm. being 1,300, 2,200, 6,000, 12,900, 14,600, and 2,600. Later it passed into a chronic condition.

Previous history.—First infected 28th February, 1935. On 14th March, 1935, parasites first appeared. Remained infected till treatment was commenced on 8th July, 1935.

(4) Cured monkey. C. 15 (*S. sinicus*).

18th October, 1935	Was infected after the previous latent infection had been cured.
21st October, 1935	Parasites first appeared.
26th October, 1935 to 31st October, 1935			Had a 'heavy infection', the parasite count on successive days per c.mm. being 3,200, 2,600, 9,200, 17,600, 20,400, and 6,600. Later it passed into a chronic condition.

Previous history.—First infected 10th June, 1935. On 21st June, 1935, parasites first appeared. Treatment commenced on 8th July, 1935.

(5) Cured monkey. C. 30 (*S. rhesus*).

18th October, 1935	Was infected after the previous latent infection had been cured.
22nd October, 1935	Parasites first appeared.
From 25th October, 1935, till its death on 30th October, 1935.			Had a 'heavy infection', the parasite count on successive days per c.mm. being 4,200, 5,000, 4,400, 19,400, and 4,700. Had been in a weak state of health and suffered from <i>Balantidium</i> infection.

Previous history.—First infected 8th May, 1935. On 15th May, 1935, parasites first appeared. Treatment commenced on 8th July, 1935.

(6) Monkey with latent infection. C. 14 (*S. sinicus*).

18th October, 1935	Was superinfected.
22nd October, 1935	Parasites first appeared.
30th October, 1935 to 2nd November, 1935			Had a 'heavy infection', the parasite count on successive days per c.mm. being 2,700, 4,900, 7,300, and 3,000. Later it passed into a chronic condition.

Previous history.—First infected on 25th March, 1935. On 8th April, 1935, parasites first appeared. Till it was superinfected, a few parasites could occasionally be seen. This monkey was never treated at any time.

(7) Monkey with latent infection. C. 18 (*S. rhesus*).

18th October, 1935	Was superinfected.
22nd October, 1935	Only a single parasite seen in the thick smear. Never had more than three parasites in thick smear at any time thereafter.

Previous history.—First infected on 23rd May, 1935. On 30th May, 1935, parasites first appeared. Had no treatment at any time.

(8) Monkey with latent infection. C. 43 (*S. sinicus*).

18th October, 1935	Was superinfected.
22nd October, 1935	Parasites first appeared. It never developed a 'heavy infection'.

Previous history.—First infected on 9th July, 1935. On 16th July, 1935, parasites first appeared. Had no treatment of any kind.

The results of this experiment may be summarized as follows:—

1. In the normal monkey there was an incubation period of three days and it took less than two more days before 'heavy infection' commenced. The 'heavy infection' stage lasted 16 days and was followed by a stage of chronic infection with parasites gradually diminishing in number.
2. All the four cured monkeys had, on an average, the same period of incubation as in the normal monkey, but the period of 'heavy infection' was considerably shorter, six days, and it took a little longer before this stage of 'heavy infection' was attained.
3. All the three monkeys with latent infection showed the slightly longer period of incubation of four days. Two of them never got a 'heavy infection' and the third, after a period of eight days, had a 'heavy infection' lasting only four days.

It is unfortunate that there was only one control monkey in this series as the course of the infection in it may not be quite typical.

EXPERIMENT WITH *P. knowlesi* INFECTION.

In this experiment there were eight monkeys in all—four cured monkeys, three *S. sinicus* and one *S. rhesus*; two with a latent infection, a *S. sinicus* and a *S. rhesus*; and two normal *S. sinicus* as controls. The infective dose of blood was drawn from a *S. rhesus* at about the height of acute infection and contained 90,000 parasites per c.mm.

The histories of the individual animals in this series are given in detail below :—

(1) Normal monkey. K. 61 (*S. sinicus*).

11th October, 1935	Infected.
15th October, 1935	Parasites first appeared.
16th October, 1935 to 20th October, 1935.			The parasite count per c.mm. on successive days was 22,100, 63,100, 36,000, 71,000, and 211,000. Died on 21st October, 1935.

(2) Normal monkey. K. 42 (*S. sinicus*).

11th October, 1935	Infected.
15th October, 1935	Parasites first appeared.
16th October, 1935 to 21st October, 1935 ..			The parasite count per c.mm. on successive days was 19,200, 16,000, 193,900, 464,000, 503,200, and 1,047,000. Died on 21st October, 1935.

(3) Cured monkey. K. 1 (*S. sinicus*).

11th October, 1935	Was infected after the previous latent infection had been cured.
16th October, 1935	Parasites first appeared. Thereafter it had a low-grade infection except for a slight rise on 22nd October, 1935, and 23rd October, 1935, to 2,700 and 900 parasites per c.mm. The animal survived.

Previous history.—First infected 15th January, 1935. Parasites first appeared in blood on 23rd January, 1935. This monkey never developed a severe infection requiring treatment with quinine. It was superinfected twice—for the first time on 23rd May, 1935, with infective blood intraperitoneally and again on 19th June, 1935, with 1 c.c. of highly infective blood by the intravenous route, but never developed a good infection. Treatment for parasitic sterilization commenced on 8th July, 1935.

(4) Cured monkey. K. 4 (*S. sinicus*).

11th October, 1935	Was infected after the previous latent infection had been cured.
15th October, 1935	Parasites first appeared. This monkey never developed a 'heavy infection' and survived.

Previous history.—First infected 31st January, 1935. Parasites first appeared in blood on 5th February, 1935. On 9th February, 1935, it was given 1 grain of quinine intramuscularly. Thereafter it had a chronic infection. 20th June, 1935—superinfected, but resisted it. Treatment for parasitic sterilization commenced on 8th July, 1935.

(5) Cured monkey. K. 36 (*S. rhesus*).

11th October, 1935	Was infected after the previous latent infection had been cured.
15th October, 1935	Smear contained a very large number of parasites—10 to 14 per field. No count made.
16th October, 1935	The count was 1,600,090 and the animal was in a very bad condition. Died on 16th October, 1935.

Previous history.—First infected on 4th June, 1935. Parasites first appeared in blood on 10th June, 1935. Treated with four injections of quinine intramuscularly for four consecutive days from 10th

772 *Absence of Effective Immunity After Cure of Protozoal Infections.*

June, 1935. Had no relapse and the animal passed into the condition of chronic infection. Treatment for parasitic sterilization commenced on 8th July, 1935.

(6) Cured monkey. K. 37 (*S. sinicus*).

11th October, 1935	Was infected after the previous latent infection had been cured.
15th October, 1935	Parasites first appeared.
16th October, 1935 to 22nd October, 1935			Had 'heavy infection', the parasite count per c.mm. on successive days being 6,400, 20,700, 17,300, 3,100, 7,000, 2,600, and 1,400. It passed into a chronic state of infection. The animal survived.

Previous history.—First infected on 29th May, 1935. Parasites first appeared in blood on 5th June, 1935. From 6th June, 1935, was given an injection of quinine daily for three days. Had two relapses on 17th June, 1935, and 29th June, 1935, and was treated with a course of quinine on each occasion. Thereafter it passed on to the stage of latent infection. Treatment for parasitic sterilization commenced on 8th July, 1935.

(7) Monkey with latent infection. K. 12 (*S. sinicus*).

11th October, 1935	Was superinfected.
15th October, 1935	Parasites first appeared.

This monkey never had a 'heavy infection'. It had been weak and emaciated for some days and died on 2nd November, 1935, but not of an acute attack of malaria.

Previous history.—First infected on 13th March, 1935. Parasites first appeared in blood on 26th March, 1935. This monkey did not develop acute symptoms requiring any quinine treatment. Passed on to a chronic state of infection.

(8) Monkey with latent infection. K. 33 (*S. rhesus*).

11th October, 1935	Was superinfected.
15th October, 1935	Parasites first appeared.
From 16th October, 1935	The parasite count gradually rose from 1,100 to 4,600, 29,100, 74,730, 76,600, and 265,300.
21st October, 1935	The animal died.

Previous history.—First infected on 23rd May, 1935. Parasites first appeared in blood on 27th May, 1935. Treatment with quinine intramuscularly was commenced from 27th May, 1935, for four days. It had three relapses during the next six weeks and required quinine injections on each occasion. Later it passed into a chronic state of infection.

The results of this experiment may be summarized as follows:—

- (1) Both the normal controls had an incubation period of four days and died on the tenth day after infection, i.e., on the sixth day after the appearance of parasites in the peripheral blood.
- (2) Of the two monkeys with latent infection, one—the *S. rhesus*—behaved in a manner very similar to the normals. It died on the tenth day after an 'incubation period' of four days. The other monkey survived.
- (3) One of the cured monkeys—*S. rhesus*—had an incubation period of less than four days and died on the fifth day of infection, much earlier than the normals. The other three survived.
- (4) One of the cured monkeys and one with latent infection—*S. sinicus*, K. 4 and K. 12—had mild attacks and survived.

DISCUSSION OF RESULTS. *

First series of experiments with monkey malaria.—In the case of *P. cynomolgi* infection there is a marked difference in behaviour between cured monkeys and those with a chronic or latent infection when inoculated with the homologous strain. The former are apparently as susceptible as normal monkeys since all became infected and with no lengthening of the incubation period, and all develop a heavy infection. Monkeys with latent infection, on the other hand, usually cannot be superinfected and do not, as a rule, develop a heavy infection. The duration of heavy infection in the cured monkeys was very uniform. The much longer duration in the one normal monkey in the series may be considered with some reserve as the average of fifteen other monkeys was eleven days. We may sum up, therefore, in the case of *P. cynomolgi* infection by saying that after parasitic sterilization the monkey is as susceptible as a normal animal, but the duration of heavy infection may be somewhat less.

In the case of *P. knowlesi* infection, the results are different. One of the cured monkeys and one of the monkeys with a latent infection showed the same susceptibility as the normal monkeys and died. The rest of the animals in both groups survived. We do not propose to discuss these results as, in the first place, the number of animals experimented with is small and, secondly, the question as to whether there is a difference in virulence of parasites in the acute and chronic stages of infection may have some bearing on the results. It is also questionable to what extent the test of cure adopted is dependable in treated monkeys. The test depended on the inability to infect a *sinicus* monkey when 2 c.c. of blood from the test animal were injected into it intraperitoneally. The more susceptible species, *S. rhesus*, would have been the animal of choice, but it was not available at the time.

Second series of experiments with monkey malaria.—In this series instead of making a comparison between cured monkeys and those with a latent infection it was decided to investigate only the question as to whether there was a residual immunity after parasitic sterilization. This also enabled us to utilize a greater number of monkeys in one series whereby the results may be considered to be less liable to error due to individual variations among the monkeys.

A total of 26 monkeys was available for the experiment, of which four were *S. rhesus* and the rest *S. sinicus*.

These were animals which had been used for testing certain anti-malarial drugs and were all in the state of chronic or latent infection. A reference to Table II will show that a considerable time had elapsed since the first infection of these animals so that they were in a condition to show whatever degree of immunity they were capable of developing. While in this condition they were treated for cure on precisely similar lines to those described as used in the previous series and the same tests of cure were employed. They were then infected with the homologous strain of monkey malarial parasite, in this case *P. knowlesi*. The results of this experiment are given in Tables II and III.

The results of this experiment indicate that monkeys which have suffered from chronic or latent infection with *P. knowlesi* and have been rendered parasitically sterile by adequate treatment show no residual immunity to infection with the homologous strain of this parasite.

TABLE II.

Showing the absence of immunity after parasitic sterilization.

Experiment with *P. knowlesi* (second series).

Monkey number.	Date of first infection.	Duration of treatment.	Date of last test for cure by inoculation of blood into susceptible monkeys.	Date of infection for test of immunity.	BLOOD EXAMINATION RESULTS.								
1	25th January, 1937.		24th June, 1937.	29th June, 1937.	3rd day—2nd July, 1937.	4th day—3rd July, 1937.	5th day—4th July, 1937.	6th day—5th July, 1937.	7th day—6th July, 1937.	8th day—7th July, 1937.	9th day—8th July, 1937.	10th day—9th July, 1937.	13th day—12th July, 1937.
2 R	"		"	"		+	+	+	+	+	+	+	+
3	"		"	"					+	+	+	+	+
5	"		"	"		+	+	+	+	0	+	+	+
6	"		"	"	+	+	+	+	+	0	+		
9	"		"	"	+	+	+	+	+	+			
10	"		"	"	+		+	+	+	+			
11	"		"	"				+	+	+			
12	"		"	"		+	+	+	+	0	+	+	
13	4th February, 1937.	15th May, 1937.	"	"		+	+	+	+	0	+	+	
14	"		"	"		+	+	+	+	0	+	+	
15	"		"	"		+	+	+	+	0	+	+	

[illegible]

	+	=	Light infection.
Note.—	++	=	Moderate infection.
	+++	=	Heavy "
++++	and	++++	

A further analysis of the results obtained in this series of monkeys is of interest.

TABLE III.

Showing the incubation period, time needed for the infection to reach a degree requiring treatment (when such a degree was reached), and the degree of infection attained in the primary infection and that induced after cure.

Monkey number.	PRIMARY INFECTION.			Severity of first relapse.	INFECTION AFTER CURE.		
	Date of primary infection.	Incubation period in days.	Severity of infection.		Date of infection after cure.	Incubation period in days.	Severity of infection.
1	25th January, 1937.	3	Heavy.	Light.	29th June, 1937.	4	Moderate.
2 R	"	4	Light.	Heavy.	"	6	Heavy.
3	"	3	Heavy.	Moderate.	"	8	"
5	"	3	"	Heavy.	"	5	"
6	"	3	"	Moderate.	"	3	"
9	"	3	"	Light.	"	4	Light.
10	"	3	Moderate.	Moderate.	"	3	"
11	"	3	"	"	"	6	"
12	"	3	"	Light.	"	6	"
13	4th February, 1937.	8	"	"	"	5	Moderate.
14	"	3	Heavy.	"	"	4	"
15	"	3	"	"	"	4	"
16	"	3	"	"	"	3	Light.
17	"	3	"	"	"	7	"
19 R	"	5	Moderate.	"	"	4	"
20 R	"	8	Light.	"	"	7	Heavy—died.
23 R	"	8	Moderate.	"	"	7	Heavy.
25	13th March, 1937.	7	Light.	"	"	3	"
26	"	6	Moderate.	"	"	3	Heavy—died.
27	"	4	"	"	"	3	Heavy.
28	"	4	Heavy.	Nil.	"	3	Heavy—died.
30	"	4	"	Light.	"	3	"
31	"	3	"	"	"	5	Moderate.
34	"	3	"	"	"	3	"
35	"	3	"	Moderate.	"	3	Heavy.
36	"	4	"	"	"	5	Moderate.
AVERAGE ..		4.11				4.5	

The results given in Table III indicate the variability in the results obtained in the primary and 'after cure' infections. In some cases the primary infection

becomes heavy sooner and is more severe than the 'after cure' infection in the same monkey and in other cases this result is reversed. In some cases a light infection in the primary attack becomes a heavy one in the after cure attack and vice versa.

These contradictory results would appear to indicate not that the effects of the primary attack exerted any influence on the after cure attack but that the latter was uninfluenced by any residual immunity and that the infection behaved as it would in a similar number of primary infections.

GENERAL SUMMARY AND CONCLUSIONS.

1. Experiments carried out with three different protozoan parasites, *Babesia canis*, *Plasmodium cynomolgi*, and *Plasmodium knowlesi*, appear to indicate that immunity to these parasites is dependent on their continued existence in the host's body.

2. Parasitic sterilization of the host is followed by susceptibility to infection by the homologous strain of parasite as complete as in a primary infection.

3. The question as to whether 'after cure' attacks are, on an average, as severe as primary attacks requires further investigation, using larger series of animals.

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INVESTIGATION ON THE ISOLATION OF THE NEUROTOXIN AND HÆMOLYSIN OF COBRA (*NAJA NAJA*) VENOM.

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IN a previous paper it has been shown by Ghosh and De (1937) that, by cataphoretic experiments under suitable conditions, more than two-thirds of the proteins associated with the neurotoxin and hæmolysin of dried cobra (*Naja naja*) venom can be removed and that partial separation of the neurotoxin from the hæmolysin can also be effected. This method, however, proved unsuccessful for further purification of these toxins. Other methods were therefore tried and these consisted in fractional precipitation of proteins by electrolytes, adsorption of the toxins by colloidal precipitates, and their subsequent elution from such precipitates, and so on. The results which have been obtained so far by adopting these procedures are recorded in this paper. From an examination of these results it will be noticed that it has been possible to separate the neurotoxin from the hæmolysin and to purify them to such an extent that for the same nitrogen content, the purified samples of neurotoxin and hæmolysin are about 17 and 4 times more active, respectively, than the dried crude venom.

PURIFICATION OF THE NEUROTOXIN.

(a) *The first stage of removal of proteins by fractional precipitation with sodium sulphate.*—The salts which are commonly used in the fractional precipitation of proteins are sodium sulphate and ammonium sulphate. In our experiments we used sodium sulphate in preference to ammonium sulphate because the latter is more toxic than the former and also because it interferes with the estimation of

proteins by the direct determination of the nitrogen content of the solution. Howe (1921) showed that the globulins present in a solution of blood serum can be precipitated when enough sodium sulphate is added to raise its concentration to 22 per cent. Ganguly and Malkana (1936) record that cobra venom contains an appreciable quantity of globulin. To separate it we gradually added, with repeated stirring, 4.4 g. of anhydrous sodium sulphate to 20 c.c. of a 0.5 per cent solution of cobra venom maintained at 37°C. The mixture was kept in the thermostat for one hour. The precipitate formed was separated from the supernatant liquid by centrifuging. The precipitate was redissolved in 10 c.c. of water and reprecipitated by the addition of 2.2 g. of anhydrous sodium sulphate and left in the thermostat at 37.5°C. for one hour. This process of solution and reprecipitation of the precipitate was repeated twice. The different fractions of supernatant liquids obtained during these operations were collected, the total volume measured, and the toxicity of the mixture determined by intramuscular injection into pigeons weighing 300 g. to 310 g. It was found that an appreciable portion of the neurotoxin was carried down by the precipitated globulin. Experiments were therefore carried out to determine the condition under which the maximum of purification with the minimum of loss of the neurotoxin occurs. It will be noticed from the data recorded in Table I that the optimum condition of precipitation is at pH 3.8. In all subsequent experiments therefore, in the first stage of purification, the globulins were precipitated at pH 3.8 with 22 per cent sodium sulphate.

TABLE I.

pH.	Percentage of protein removed by precipitation.	Percentage of neurotoxin lost.
3.8 ..	68.7	15.1
5.8 ..	69.1	18.2
7.0 ..	69.7	19.7
9.4 ..	70.1	21.2

(b) *The second stage of removal of proteins by fractional precipitation with sodium sulphate.*—Forty c.c. portions of the cobra venom solution from which the globulins had been removed by treatment with 22 per cent sodium sulphate at pH 3.8 were taken in a number of conical flasks. Their pH was adjusted to different values and then increasing amounts of sodium sulphate (anhydrous) were added to these solutions and the mixtures kept at 37.5°C. for one hour. In each case the precipitate formed was separated from the supernatant solution by centrifuging. The precipitate was redissolved and reprecipitated as described in the first stage. The different fractions of supernatant liquid were collected, mixed, and made up to

100 c.c. The protein content and the toxicity of the mixture were then determined. It was found in this way that the optimum condition of precipitation was in the neighbourhood of pH 5.8, the sodium sulphate concentration in the solution being 33 per cent. The supernatant liquid under this condition of precipitation was very rich in neurotoxin and poor in protein. This will be evident from the data recorded in Table II and obtained from an analysis of the supernatant liquid. In all subsequent experiments therefore the second stage of fractional precipitation of proteins was carried out in the neighbourhood of pH 5.8 with 33 per cent concentration of sodium sulphate.

TABLE II.

Number of M. L. D. per mg. of N₂ of the dried crude venom : 21.9.

pH.		N ₂ in mg. per 100 c.c. of solution.	Protein per 100 c.c. of solution.	Number of M. L. D. per 100 c.c. of solution.	Number of M. L. D. per mg. of N ₂ in the solution.
3.8	..	0.874	5.463	185	224
5.8	..	0.755	4.719	175	232
7.0	..	0.652	4.075	150	230
9.4	..	0.566	3.530	120	212

(c) *The third stage of purification by adsorption on tungstic acid and elution from its surface.*—To 60 c.c. of the supernatant solution obtained after the second stage of purification was added 0.5 c.c. of 10 per cent sodium tungstate and 0.5 c.c. of 2/3 normal sulphuric acid, and the mixture kept at 37°C. for ten minutes. The precipitate was then separated from the supernatant liquid by centrifuging and washed twice with an aqueous solution containing sodium tungstate and sulphuric acid in the same proportion as already described. The precipitate was then treated with 10 c.c. of water and N/5 alkali was added to it drop by drop with repeated stirring until the pH was 8.0. The tungstic acid precipitate dissolved at this pH. The solution was then treated with a slight excess of barium chloride to remove the tungstic acid as barium tungstate. The excess of Ba ions was precipitated by treatment with the requisite quantity of Na₂SO₄. The nitrogen content and toxicity of the supernatant solution were determined. The results obtained by repeating the experiment a number of times using the same sample of cobra venom are recorded in Table III. It will be noticed that the results are fairly reproducible. The data in column 5, under the heading 'purification effected', are obtained by dividing the corresponding figures in column 4 by 21.9 which is the number of M. L. D. per mg. of nitrogen of the dried crude venom. It will be noticed that

the neurotoxin has been concentrated in a protein fraction in which its activity, for the same nitrogen content, is 15 times greater than that of the dried crude venom. This active protein fraction does not contain haemolysin.

TABLE III.

Experiment number.	N ₂ in mg. per 10 c.c. of solution.	Number of M. L. D. in 10 c.c. of solution.	Number of M. L. D. in 1 mg. of N ₂ .	Purification effected.
1	0.50	165	330	15.06
2	0.424	140	330	15.06
3	0.478	160	334	15.25
4	0.508	170	334	15.25

(d) *Removal of electrolytes from the purified neurotoxin fraction.*—The solution obtained after the removal of tungstic acid contains electrolytes in addition to the neurotoxin which is very probably still associated with an appreciable quantity of inert protein. The solution was therefore dialysed in a cellophane bag against distilled water inside a refrigerator. When the water outside the bag ceased to give any test of sulphate the solution was withdrawn from the bag, its toxicity tested, and a measured volume was evaporated in a vacuum desiccator. When completely dry the residue was weighed. It was found that 12.3 mg. dry residue contained 625 M. L. D. of neurotoxin. Therefore 1 mg. of the residue contained 50.3 M. L. D., whereas 1 mg. of the dried crude venom contained 3.33 M. L. D. Therefore a purification of about 15.3 times of the neurotoxin fraction has been effected.

PURIFICATION OF THE CONCENTRATED NEUROTOXIN FRACTION BY CATAPHORESIS.

The 15 times purified neurotoxin fraction obtained after dialysis was subjected to cataphoretic experiments with the object of purifying it still further. The apparatus and arrangements used were the same as described in a previous paper (Ghosh and De, *loc. cit.*). Ten c.c. of the neurotoxin solution containing 200 M. L. D. were adjusted to the requisite pH and were placed in the anode chamber of the cell. The anode chamber was separated from the middle one by a parchment membrane and between the cathode chamber and the middle one a membrane of cellophane was used. About 216 coulombs of electricity were passed through the cell in the course of six hours. After this the toxicity and nitrogen content

of the middle chamber were estimated. The results are recorded in Table IV. It will be noticed from these data that a further purification of the neurotoxin fraction is possible and the range of optimum pH is 8.0 to 9.0.

TABLE IV.

Number of M. L. D. per mg. of N₂ of dried crude venom : 21.9.

pH.	N ₂ in mg. in the middle chamber.	Number of M. L. D. in the middle chamber.	M. L. D. per mg. of N ₂ in the middle chamber.	Purification effected.
4.2 ..	0.114	40	350	16.0
6.0 ..	0.092	33	358	16.4
8.0 ..	0.067	25	373	17.0
9.4 ..	0.046	17	370	16.9

(e) *Purification by adsorption on and elution from the surface of silica gel.*—It was found in a preliminary experiment that silica gel adsorbed the neurotoxin of the cobra venom and that the adsorbed neurotoxin can be partially extracted by treatment with hydrochloric acid solution. An attempt was therefore made to further purify the concentrated neurotoxic fraction obtained after the stage (c), i.e., after the removal of tungstic acid as barium tungstate, by adsorption and elution from silica gel. Twenty-five c.c. of the neurotoxin fraction containing 200 M. L. D. were adjusted to pH 9.6 and shaken for 30 minutes with 5 c.c. of a silica suspension containing 104 mg. SiO₂. The silica was separated from the supernatant solution by centrifuging. The solid was washed once with 10 c.c. of water adjusted to pH 9.6 and then shaken with 15 c.c. of N/2 hydrochloric acid solution for 30 minutes. The mixture was then centrifuged and the supernatant solution withdrawn and analysed. It was found to contain 0.242 mg. N₂ and 88 M. L. D. of neurotoxin. Therefore compared to the crude cobra venom it was 16.6 times purer.

(f) *Iso-electric behaviour of the 15 times purified neurotoxin fraction.*—In a previous paper it has been stated by us (*loc. cit.*) that the neurotoxin is a substance pronouncedly basic in character. The experiments therein recorded were carried out with the crude cobra venom. It was therefore thought desirable to verify and extend our previous observation with the highly purified neurotoxin sample now prepared. The experimental arrangements were exactly the same as described in the paper referred to. The pH of the solution containing the neurotoxin was adjusted to the requisite value and the solution was placed in the middle chamber

of the glass cell. The middle chamber was separated from the two side chambers by parchment membranes. After passing 216 coulombs of electricity in the course of six hours the contents of the anode and the cathode chambers were tested for neurotoxin by intramuscular injection into pigeons. The results are recorded in Table V. It will be noticed that the iso-electric point of the neurotoxin lies above pH 9.4. It is perhaps in the neighbourhood of pH 11.0.

TABLE V.

pH.	Cathode chamber.	Anode chamber.
7.0 ..	+	—
8.0 ..	+	—
9.4 ..	+	—
11.0 ..	—	—

PURIFICATION OF THE HÆMOLYSIN.

It has been observed by us (*loc. cit.*) that the major portion of the hæmolysin can be separated from the neurotoxin, when sufficient quantity of sodium chloride is added to a one per cent solution of cobra venom, so as to bring the final concentration of the salt to 20 per cent. Ganguly (1937) has also observed that the entire amount of hæmolysin free from neurotoxin is found in the precipitate formed by saturation with sodium chloride. The hæmolysin fraction thus obtained is still associated with a large amount of inert protein. Attempts were therefore made to purify it still further. After a number of preliminary experiments the following method was adopted to remove some of the protein impurities: The precipitate obtained in a one per cent cobra venom solution, containing 20 per cent sodium chloride and kept at 37°C. for one hour, was separated from the supernatant solution by centrifuging. The precipitate was then dissolved in 10 c.c. of water and the reaction of the solution adjusted to pH 7.8. The solution contained in a test-tube was placed in a water-bath at 86°C. After two minutes the solution became turbid, when one drop of N/5 NaOH was added. The solution was stirred to prevent the formation of clumps and two minutes later another drop of the NaOH solution was added. After six minutes the test-tube was withdrawn from the bath and the supernatant solution separated from the precipitate by centrifuging. The supernatant solution contained the hæmolysin. On analysis it was found that its hæmolysin and protein contents were, respectively, 71 per cent and 19.9 per cent of those present in the material with which we started. Therefore compared to the original venom, the concentrated hæmolysin fraction is 71/19.9, i.e., about 3.6 times purer than the crude venom. The hæmolysin thus purified was freed from electrolytes and was

subjected to cataphoretic experiments for further purification. The experimental arrangement was the same as described under Section (c). After passing 216 coulombs of electricity through the cell in the course of six hours, the content of the middle chamber to which the hæmolysin migrated was analysed. The results are recorded in Table VI. It is assumed that per mg. of N_2 of the crude venom 16.6 units of hæmolysin are present. It will be noticed that the maximum purification is four times.

TABLE VI.

pH.	Mg. of N_2 per 10 c.c. of solution of the middle chamber.	Number of units of hæmolysin per 10 c.c. of solution in the middle chamber.	Purification effected.
6.0 ..	1.32	85	3.9
8.0 ..	0.9	60	4.0
9.4 ..	0.48	32	4.0

DISCUSSION.

The separation of the neurotoxin and the hæmolysin of cobra (*Naja naja*) venom from one another and also from the associated non-toxic protein material has previously been attempted by a number of workers. Of these Ganguly (*loc. cit.*) and Ganguly and Malkana (*loc. cit.*) appear to be the most successful. They succeeded in concentrating the neurotoxin within a protein fraction representing 16.8 per cent of the dried crude venom. We have, however, been able to concentrate the neurotoxin within a protein fraction 5.2 per cent of the dried crude venom. This shows that the sample of neurotoxin separated by us is more than three times more active than the sample obtained by Ganguly and Malkana. It appears therefore that for the same nitrogen content, the sample of neurotoxin separated by us is the most active. The same remark also holds for the concentrated hæmolysin fraction obtained by us.

CONCLUSION.

1. A very active sample of cobra (*Naja naja*) neurotoxin free from hæmolysin has been prepared by fractional precipitation with sodium sulphate, followed by adsorption on and elution from tungstic acid surface, dialysis, and finally by cataphoresis. For the same nitrogen content the sample was 17 times more toxic than the dried crude cobra venom.

2. The iso-electric point of the purified neurotoxin appears to be above pH 9.4.

3. The hæmolysin of cobra (*Naja naja*) venom has been separated from the neurotoxin by precipitation with sodium chloride and then further purified from the greater portion of the other proteins by coagulating them at 86°C. from a faintly alkaline solution, and finally by the cataphoresis of the solution obtained after heat coagulation. For the same nitrogen content the hæmolysin fraction so purified is four times more active than the crude venom.

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THE SUCCESSFUL TRANSMISSION OF CUTANEOUS LEISHMANIASIS BY INOCULATION TO MAN FROM A NATURAL LESION OCCURRING ON A DOG IN INDIA.

BY

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LESIONS due to cutaneous leishmaniasis occurring naturally on the nose of a dog living in the Punjab, India, were recorded by Sinton and Shortt (1934). At the time when the nature of the infection was first recognized, infected material was inoculated into a monkey (*Silenus rhesus*) and into two human volunteers.

The monkey was inoculated by cutaneous scarification (eyebrow), and also by intradermal and subcutaneous injections (abdomen). An Indian volunteer, who had a history of oriental sore in childhood, was inoculated by cutaneous scarification on the front of the forearm, and a similar inoculation was made in a European, who had not previously suffered from any form of leishmaniasis. The material used for scarification was a serosanguineous exudate from a small ulcer on the nose of the dog. This material showed Leishman-Donovan bodies on microscopical examination, and a small drop was scarified into the skin with a Hagedorn's needle in a manner similar to smallpox vaccination. The sites of inoculation were in every instance carefully noted and accurate measurements made of their position.

The early after-histories of these cases have been reported by Shortt, Sinton and Swaminath (1935). The monkey died of pneumonia about 6 months after inoculation, having shown no signs of leishmania infection. Neither the Indian nor the European showed any lesions at the end of the same period.

The period of observation was continued, but although the Indian showed no signs of infection up to the end of 20 months the European did.

Careful examination of the site of inoculation in the latter case revealed nothing abnormal after 12 months, but 10 days later a very minute reddish spot was noticed,

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which was at first thought to be due to an insect bite. As it had not disappeared 7 days later, its surface was gently scarified and a small drop of serum expressed. This was found to contain innumerable Leishman-Donovan bodies, and a culture was made successfully on N.N.N. medium.

The lesion at this time was a slightly raised, pinkish point about 1 mm. in diameter with a lighter pink areola. The surface was unbroken, and the spot would have attracted little attention but for its persistence and the fact that its position coincided so exactly with the point of inoculation.

The later growth of the lesion was very slow. At the end of 6 months, its appearance was that of a small, flattened hemispherical, intracutaneous nodule, about 4 mm. in diameter and projecting above the surrounding skin. It was freely movable on the subcutaneous tissue and resembled a small keloid growth. The overlying skin was smooth with a light reddish-purple appearance. Occasionally a few dry whitish scales were present on its surface, usually after it had been scarified to obtain infected material.

It reached its maximum size at about 12 months after its original appearance. At this time its colour was slightly more purplish, its superficial diameter about 5 mm., its consistency more solid on palpation, and its surface more raised and covered with a few yellowish-white scales, but no ulceration was present.

At about 10 months, a small nodule about 2 mm. in diameter was detected by palpation about 20 mm. external to the primary lesion and slightly proximal to it. This nodule was attached to the skin and was freely movable on the subcutaneous tissue. It never appeared to get any larger nor was it detectable on surface inspection. At no stage of the infection could any enlarged lymphatic glands be detected.

As the result of injury at about the 15th month, a small hæmorrhagic bulla formed on the surface of the primary lesion. This burst leaving a small superficial ulcer which healed very quickly. This was the only occasion when any ulceration occurred. From this time onwards until it disappeared, the lesion did not increase in size, but was covered with a very thin, scaly crust of a yellowish-white colour.

Numerous leishmania were detectable both by smears and by culture up to the end of about 16 months, but could not be found after about 18 months. At this time the lesion began to become less prominent, and at the end of 20 months it had healed completely, leaving a very slightly depressed, purplish-red scar about 4 mm. in diameter, surrounded by a slightly redder areola about 2 mm. wide. The secondary nodule had disappeared about 2 months earlier.

The lesion persisted for a total of 20 months and healed spontaneously. At no time was any dressing applied to it, nor was any medical treatment given.

Certain cases of oriental sore have been reported in which fever was recorded during the incubation period. It was suggested that this might be due to a general infection with the parasite prior to the appearance of local manifestations*. The patient in the present case had some fever of an 'influenzal' nature a few months after inoculation, but blood cultures made for the detection of leishmania revealed no parasites nor did microscopical examination of the blood.

* References to such cases are given by Sinton (1925).

At the end of about 14 months from the appearance of the lesion, another human volunteer was inoculated from it. After an incubation period of 5 months, a small spot appeared at the point of inoculation. This resembled the early primary lesion seen in the original volunteer, and showed a fair number of parasites. The papule has developed after 4 months into a slightly raised, pinkish, keloid-like nodule, about 5 mm. in diameter, with no signs of ulceration but with a few whitish scales on the surface.

These experiments show that, although canine leishmaniasis appears to be a rare disease in India (*vide* Sinton and Shortt, *loc. cit.*), yet the cutaneous form of the disease is transmissible to man by experimental inoculation.

Apart from the transmission of the infection, an interesting feature of this case is the very prolonged incubation period of more than 12 months. The incubation period in the naturally acquired human disease is usually said to be about 3 months, or at the outside 6 to 7 months. Napier and Halder (1936) have, however, estimated an incubation period of more than 3 years in a case recently seen by them in Calcutta.

One must wonder, therefore, what happens to the parasites during this long period of apparent quiescence. The fact that the lesion in our primary case appeared at the exact site of inoculation suggests that the parasites were localized there during the interval, and had not multiplied in sufficient numbers, or with sufficient virulence, to produce any local lesion detectable on naked-eye examination. These results show that, in attempts at the experimental transmission of cutaneous leishmaniasis by any suspected insect vector, a very long period of observation is necessary before the results can be declared to be negative.

SUMMARY.

A European volunteer has been given cutaneous leishmaniasis by the inoculation of infected material obtained from a natural case of this disease, occurring in a dog in India.

The incubation period of the lesion was over 12 months, and the nodule persisted for 20 months. Healing was spontaneous without any therapeutic intervention.

The infection was transmitted by cutaneous scarification from the first to a second European volunteer, in whom the incubation period was only 5 months.

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STUDIES IN 'EFFECTS OF HEAT'.

Part I.

BIOCHEMICAL AND PHYSICAL CHANGES IN TEN CASES SUFFERING FROM 'EFFECTS OF HEAT'.

BY

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INTRODUCTION.

THIS work was undertaken during the hot weather of 1936 with a view to studying the biochemical and physical changes in blood and urine in 'effects of heat' cases and their possible correlation with the clinical conditions of the patients.

With the consent of the army authorities 19 stations were selected to supply the necessary material. In addition some civil hospitals agreed to co-operate.

Sterile stoppered bottles containing adequate anti-coagulants for blood samples and sterile stoppered bottles for serum samples were supplied to all hospitals concerned.

Anti-coagulants used for blood samples were:—

(i) *Potassium oxalate*.—In one set of sample bottles this was added in quantities to give a concentration of 2.5 mg. of potassium oxalate per c.c. of blood, provided 5 c.c. were introduced, and

(ii) *Ammonium citrate*.—This was made by Folin's (1934) technique. Of this 0.05 c.c. was added to the other set of sample bottles.

Solutions of anti-coagulants were made and the appropriate quantities added to the bottles. The moisture was evaporated under reduced pressure, leaving a film on the inner walls of the bottles.

The hospitals were requested to draw under aseptic conditions 20 c.c. samples of blood from cases soon after their admission and to introduce 5 c.c. to each of the two bottles containing the different anti-coagulants. The remaining 10 c.c. of blood were to be used in obtaining serum samples. Three such sets of samples were required to be sent from each patient, the first set on the day of admission, the second on the following day, and the third during convalescence. For fatal cases it was requested that an additional sample be drawn either just before death by veni-puncture, or just after death by heart puncture.

Twenty-four hours' collections of urine from each patient on every alternate day were also required to be sent in sterile stoppered bottles. An adequate supply of formalin preservative was given and the hospitals were asked to add 60 minims for 24-hours' collections or two minims for each ounce of urine.

A further request was made to forward complete case notes, temperature and diet charts, and to fill in the special forms provided. These latter included information regarding:—

1. Details of duty performed by the patient and clothing used three days prior to admission.
2. Consumption of alcohol.
3. History of previous febrile conditions or any disturbances due to heat.
4. Behaviour of the patient a week prior to the attack, and any other relevant information.

TECHNICAL DETAILS.

(1) *Biochemical.*

In view of the meagre data available regarding the biochemical changes in human cases suffering from the 'effects of heat' it was decided to do as complete analyses of blood and urine samples as possible in the hot weather of 1936, and to concentrate only on the more significant changes during the subsequent investigations.

Table I gives the analytical methods employed.

(2) *Physical.*

The details and methods followed in obtaining the physical data are given in *Appendix A*.

DISCUSSION.

The total number of cases investigated by us was only ten. The case incidence had been unusually low owing to the early onset of the monsoon in 1936. The total numbers of blood and urine samples received were fifteen and fourteen respectively. In no case was the number of samples required by the instructions available.

In view of the anticipated continuance of the work during the next hot weather only a preliminary discussion is being attempted in this communication.

NITROGENOUS CONSTITUENTS.

Table II (A and B) gives the figures for the N.P.N. constituents of blood and urine.

More than one sample of blood was available from only four cases (cases 2, 7, 8, and 10); and of urine from two cases (cases 2 and 7). An increase in the N.P.N. constituents of blood was found in every case, and as would be expected a diminution coincided with the recovery of the patient. On the other hand, case 10 which ended fatally gave the highest N.P.N. figure in our series. The second sample of blood from this patient (CB₃) drawn about half an hour before death had 140 mg. of N.P.N. per 100 c.c. of blood. Hall and Wakefield (1927) in their experimental study on dogs and Marsh (1930-31) using rabbits have also noted a high blood N.P.N.

It is well known that in pyrexia cases the rate of destruction of proteins is abnormally high, being directly proportional to the temperature of the patient. Voit (1895) (quoted by Lusk, 1919) explains this increase in protein katabolism as being due to a rapid combustion of available glycogen and consequent utilization of proteins for energy needs.

NITROGEN RETENTION.

In hyperthermia unassociated with obvious renal complications the usual finding is an increased excretion of the urinary nitrogenous constituents in an attempt to lower the blood N.P.N. to the normal level. In the majority of cases in our series a definite tendency towards the retention of these constituents in the blood was noted.

A study of five urine samples (UB₂, I—V) received from the same case (case 2) showed a minimum urinary nitrogen excretion of 1.575 g. in 24 hours in the second sample (UB₂, II). With the progress of the case towards recovery the total nitrogen excretion gradually increased to 7.140 g. in the last sample (UB₂, V).

RENAL INSUFFICIENCY.

This tendency towards N.P.N. retention along with the results of examination of the catheter-drawn urine sample collected a couple of hours before death in case 10 suggests the presence of renal insufficiency, at least in severe cases of heat stroke. The urine in this case had about 0.4 per cent of albumin and a variety of urinary casts, blood, and acetone. Gauss and Myer (1917) observed numerous hyaline and granular casts in the urine of their cases on the second day. Marsh (1933) also noted the presence of sugar, acetone, a few hyaline casts, and some red cells in the urine of the case reported. Gradwohl and Schisler (1917) noted a similarity between urine and blood chemical reactions both in heat and renal insufficiency cases.

Further evidence of the presence of renal insufficiency is available from our findings in the three fatal cases of our series (cases 1, 9, and 10). The blood calcium figures in these cases were low. The second sample of blood from case 10 had the lowest calcium figure noted by us (6.9 mg. per 100 c.c.). Samples from the two local cases (cases 9 and 10) which ended fatally had high inorganic phosphate contents as well. Creatinine figures of 4.215 mg. and 2.80 mg. per 100 c.c. of blood in cases 1 and 10 respectively are also suggestive.

The evidence thus available suggests that renal damage may be one of the complications in severe cases of heat stroke.

Table III (A and B) gives the acid and base radicals of blood and urine samples.

CHLORIDES.

A good deal of discussion has already centred round the chloride contents of blood and urine in 'effects of heat' cases. Marsh (1933) has recorded a case with 263 mg. of chlorides as NaCl per 100 c.c. of blood and the urinary chloride absent in the 24-hours' urine, on the first, second, and third days. Our cases showed a similar state of affairs. The lowest blood-chloride figure amongst the cases in our series was 314 mg. per 100 c.c. of blood, noted in the case of heat exhaustion studied by ourselves (case 1).

Urinary chloride excretion in 24 hours had been low in every case we have studied. In some (cases 6, 7, and 8) the low figures were found in spite of the excessive quantity of urine passed in 24 hours. In addition the specific gravity and total base figures of the samples were low, suggesting a poor concentration of solids. A scrutiny of the treatment charts showed administration of chlorides as salines in cases 3, 6, and 10 only; others received no saline treatment. Most of the patients were allowed fluids *ad lib.*, but owing to there being no administration of salts the fluids were not retained by the patients, the depletion of chlorides from the plasma having caused a fall in the osmotic pressure of blood (Haldane, 1929). This obviously accounts for the excessive quantity of urine with low solids, including chlorides; although Ambard (1920) thinks that the excretion of chlorides through the kidneys depends more upon the needs of the organism than upon their concentration in the plasma.

In all the cases of our series with more than one sample of blood or urine, a higher value for blood and urinary chlorides was noted in cases ending with recovery. Case 2 is particularly interesting, since two blood and five urine samples were received from this patient. His second sample of urine (UB₂, II) showed a minimum chloride excretion of 0.105 g. in 24 hours with a total quantity of 875 c.c. urine. As already mentioned the same sample also gave the minimum nitrogen figure. A gradual rise in chloride contents was noted in subsequent samples.

Ample evidence is thus available that, despite the low excretion of chlorides in the urine, the blood chlorides are also at a low level in 'effects of heat' cases.

Normally, blood contains about 500 mg. of chlorides as NaCl per 100 c.c. and the tissues contain varying quantities. Salt when ingested passes almost unabsorbed through the stomach and small intestines, the absorption being mainly in the colon. During the process of digestion chloride ions are excreted into the stomach as hydrochloric acid with a potential loss of blood chlorides which persists until re-absorption has taken place in the colon (Trusler, 1928). Normally the excretion of chlorides per rectum is of a low order, unless the individual is suffering from diarrhoea or some such disorder which prevents re-absorption of chlorides in the colon.

The natural means of chloride loss from an organism are through the skin as sweat, the kidneys as urine, and the rectum as faeces, the last being of minor importance.

In our series of cases it is regretted that no faecal-chloride estimations were done; further work in this connection will be taken up during the next summer. It may, however, be noted that none of our cases suffered from diarrhoea, hence it is unlikely that faecal excretion of chlorides played any important rôle in producing hypochloræmia. Vomiting, another clinical condition to cause chloride loss, was not a feature in any of our cases.

It has already been shown by us as well as by other workers that the urinary chloride figure is low in 'effects of heat' cases, hence the hypochloræmia could not have been the result of excessive loss through the kidneys.

In the absence of data showing the actual chloride excretion through sweat in our series, it is difficult to assess how far loss of salt through the skin had been responsible in lowering the blood chlorides. All our cases except case 1 were diagnosed 'heat stroke', a condition in which sweating is not a feature.

Marsh (1933) made a study of normal persons in Persia month by month from March to August and came to the conclusion that at the end of summer the bodily stocks of sodium chloride are rather low. A similar series of observations is in progress in this Laboratory which will form the subject of a future communication. During the summer in the tropics a certain amount of chlorides is lost from the body by sweating. Moss (1923-24) found that a man may excrete as much as 2 g. to 3 g. of chlorides, as NaCl, per hour in sweat during hard labour at temperatures of 98°F. to 102°F. The dehydration resulting from sweating is, however, said to cause a fall of only 1 to 2 millimoles in plasma-chloride concentration, a figure which appears to be rather low to account for the extent of hypochloræmia noted by us.

None of our cases except perhaps two (cases 7 and 8) could be said to have been engaged in duties requiring hard physical labour. These two patients as defaulters were engaged on fatigue duty prior to the attack. The blood-chloride figures in these two cases were, however, by no means the lowest observed by us.

Only two of our cases (cases 3 and 7) gave a history of suppression of sweating, 18 and 72 hours before the attack, with blood chlorides of 415 mg. and 360 mg. respectively in their first samples of blood. Marsh (1930-31) concluded that the critical blood-chloride level to cause depression of sweating lies somewhere between 436.7 mg. and 500 mg. per 100 c.c. of blood. If this is true the blood-chloride level in all other cases of our series, in the absence of any history of suppressed sweating, is likely to have been above 436.7 mg. per 100 c.c. at the time of attack. The average figure for blood chloride of the first blood samples in our series of cases was 372.9 mg. per 100 c.c. It is interesting to note that the maximum figure (415 mg.) amongst first blood samples was noted in case 3, one of the two cases giving a history of suppressed sweating. The first blood sample of the other case (case 7) had 360 mg. of chlorides as NaCl per 100 c.c. of blood, a figure also noted in other cases without any history of suppressed sweating. Our preliminary findings, therefore, do not seem to be in agreement with Marsh's conclusions.

None of our cases gave any history of excessive sweating during the period preceding the attack. Moreover, it appears to us likely that if the hypochloræmia observed had been of some standing, nature would, in all probability, have tried to

compensate by increasing the desire for salt intake rather than leave the individual a subject susceptible to heat stroke. There is no evidence available to suggest any increased craving for chlorides amongst our cases.

It is thus possible that sweating and other natural means of chloride loss are not solely responsible in causing hypochloræmia to the extent noted in heat stroke cases. None of our cases ending in recovery, from whom more than one sample of blood was received, was treated with salines; although their later samples showed increased chloride contents. On the other hand, the second sample of blood (CB₃) from case 10, a fatal case, had a lower blood-chloride figure than the first sample (CB₂) in spite of abundance of chlorides having been administered to this patient.

It appears to us difficult to reconcile these conflicting data unless we find some other contributory factor responsible in causing hypochloræmia.

DONNAN'S EQUILIBRIUM.

Donnan pointed out that if a colloidal solution such as a sodium salt of congo red is enclosed in a parchment bag, the sodium ions diffuse out, whilst the colloidal part of the salt does not. His law enunciates that in the presence of an indiffusible colloid such as protein on one side of a membrane, the electrolytes diffuse through the membrane until an equilibrium in their distribution on both sides of the membrane has been effected.

A similar phenomenon is observed in the distribution of the two monovalent anions Cl' and HCO'₃ in cells and plasma. When the system is at equilibrium their concentration should thus be:—

$$\text{HCO}'_3 (\text{cells}) : \text{HCO}'_3 (\text{plasma}) :: \text{Cl}' (\text{cells}) : \text{Cl}' (\text{plasma}).$$

Any increase in HCO'₃ in cells naturally unbalances this equilibrium. To restore the balance, diffusion of HCO'₃ from the cells into the plasma takes place, closely followed by an equivalent chloride diffusion in the opposite direction until the equilibrium is restored.

Normally the concentration of chlorides in the blood cells is only half of that in the plasma. The interchange, referred to above, would tend to equalize the chloride concentration in both the phases causing an increase in the chloride content of the cells. The excess of HCO'₃ in plasma combines with sodium, increasing the NaHCO₃ concentration and thus diminishing the acidifying effect.

The acid-base balance of the organism is thus maintained within narrow limits. The chlorides, owing to the cell boundaries in general being pervious to them and since they make up about two-thirds of the anions in the plasma, form a very important electrolyte in maintaining this equilibrium.

LACTIC ACID.

Muscular exercise or excessive tissue activity increases the production of lactic acid in the tissues. During hyperthermia, the metabolism increases and it has been shown that for every rise of 1°F. in the internal temperature of the body the basal metabolism increases by 7.2 per cent (Wright, 1931).

Hall and Wakefield (*loc. cit.*) as well as Marsh (1930-31) in their studies of experimental heat stroke in animals noted high blood lactic acid. In our series a similar increase has been found. The second sample of blood (CB₂) from case 10, a local case ending fatally, in which the estimation of lactic acid was done within an hour of collection showed 42.48 mg. of lactic acid per 100 c.c. or 4.7 millimoles per 1,000 c.c., a figure more than double the normal upper limit of lactic acid in the blood.

The acidæmia resulting from the accumulation of lactic acid in the plasma in heat stroke cases should also encourage pulmonary ventilation, a physiological means of combating acidæmia, producing a low CO₂ tension in blood. Hall and Wakefield (*loc. cit.*) as well as Marsh (1930-31) have noted a low plasma CO₂ tension in their series of experimental animals. In our next series of cases we hope to try to confirm this.

It is not known whether chlorides also can take part in combating lactic-acid acidæmia in order to maintain the acid-base balance, in the same way as they do in the case of acidæmia due to HCO₃' increase in the cells. If both chlorides and lactic-acid ions can permeate tissue cell walls, Donnan's equilibrium should as a consequence result with the transfer of chlorides from the blood to the tissues in order to counterbalance the anion increase in blood.

It has been noted that during starvation and diabetic acidosis the organic acids formed replace HCO₃' and Cl' in the blood. The permeation of lactic-acid ions through tissue cells is known to occur, but the evidence regarding chloride ions is conflicting. Banus and Katz (1927) found that by perfusing muscles with blood containing HCl there was no change in the Cl' content of the perfusate. How far such experiments do actually represent the true state of affairs in a living organism is open to question. Katz (1896) found the muscle chlorides in a case of suicide to be about one-sixth of that in the blood serum. Chlorides are the most abundant electrolytes in the blood and they have been found in all the cells and fluids of the body; it is, therefore, unlikely that cell walls are not at all permeable to chloride ions. However, no evidence is so far available to show that in hyperthermia cases there is an increase in tissue chlorides. Neither Hall and Wakefield (*loc. cit.*) nor Marsh (1930-31) in their series of experiments estimated the muscle chlorides in animals.

Further, a marked increase in the undetermined anions was noted by us, the concentration becoming less with recovery. Whatever the nature of these may be, they must play a part in increasing the acidæmia.

HYDROGEN ION CONCENTRATION.

Hall and Wakefield (*loc. cit.*) as well as Marsh (1930-31) in their animal experiments reported a change in the blood pH to the acid side.

It is likely that, owing to the excessive presence of organic-acid anions in human cases, a similar state is present. Studies in this connection are in progress here.

It is well known that the stability of proteins as colloids is dependent on the electric charge carried by the molecules. Any shift of the 'charge' towards the

iso-electric point changes its properties so much that at the iso-electric point (about pH 5.0) they are precipitated easily. If a shift in the pH of blood towards the iso-electric point occurs in human cases suffering from the 'effects of heat', a change in the cellular wall proteins appears to be possible. This, along with the effect of hyperthermic blood, may alter the normal properties of the cell walls.

Peters *et al.* (1926) have shown that venous stasis lowers the plasma-chloride concentration owing to the passage of chlorides to the tissues. During hyperthermia a dilatation of the capillaries is present, which augmented by the increased viscosity of blood as noted by us (*see* Table IV) would tend to produce a relative venous stasis.

The suggested change in the properties of the cell walls, coupled with the effects of venous stasis, may upset the normal conditions and increase the permeation of the chloride ions through the tissue cells.

It is thus likely that a contributory factor to hypochloræmia in heat stroke cases is the increased concentration of lactic-acid and other anions, which upset the acid-base balance of the organism. The chlorides being the most abundant anions in the normal plasma would naturally be the first to be affected and their shift from plasma to tissues is likely to occur, resulting in hypochloræmia, in order to establish the 'Donnan's equilibrium' and thus maintain the acid-base balance of the organism.

A study of Table III (A) would show that an inverse relationship between blood-chloride and lactic-acid figures is indicated in cases where more than one sample was analysed, lending support to the view that elimination of organic anions tends to restore the chlorides to the normal level.

Table III (A and B) further shows that there is a tendency to the retention of total bases in the blood as evidenced by the figures for blood and urine samples. These were generally high in blood and low in urine. In view of the accumulation of anions in the blood it is not surprising that the bases were retained in an attempt to maintain the acid-base balance.

BLOOD SUGAR.

Marsh (1933) has reported an increase in blood sugar in his heat stroke case. In our series blood sugars were estimated only in the local samples, CB₁, CB₂, and CB₃; the figures were 135.5 mg. and 297.4 mg. per 100 c.c. of blood respectively in the first two samples; CB₃, the second sample of blood from case 10, which was drawn about half-an-hour before death, showed only a trace of sugar. Our figures, therefore, support Marsh's findings. The reason for the marked diminution of blood sugar in CB₃ is not quite understood, unless it was due to an increased demand by the organism for readily combustible material as a result of hyperthermia and the excessive muscular spasms from which the patient suffered during the last few hours.

PHYSICAL PROPERTIES.

Table IV gives some of the physical properties of citrated blood samples, viz., specific gravity, viscosity, electrical conductivity, and surface tension.

In view of the small number of samples thus analysed a detailed discussion of the physical properties in 'effects of heat' cases and their correlation with severity of attack and biochemical changes is not possible at this stage of the work.

From the preliminary observations, however, it is seen that the viscosity and specific gravity of the blood are generally higher in severe cases; cases 3, 4, 5, 7, 8, and 10 showing high viscosity figures had one or more signs or symptoms indicating the severity of the disease, e.g., cyanosis, extreme restlessness, loss of consciousness, suppression of sweat, cramps, etc. (see *Appendix B*). There is also an indication of a general lowering of the surface tension of the blood in such cases.

It appears, therefore, that owing to the loss of moisture, protein and possibly lipid substances in the blood were appreciably increased causing a rise in viscosity and density with lowering of the surface tension. This conclusion is generally supported by estimations of total proteins and cholesterol in sera (see Table IV). Samples B₅, B₆, B₉, and B₁₂ all had high viscosities, low surface tensions, and raised protein contents, and sample B₆ a high cholesterol figure as well. No serum was available from sample B₃ which had rather a high viscosity figure so that no protein or cholesterol estimations were possible. It is, however, interesting to note that this patient suffered from suppression of sweating 18 hours before admission to hospital; the high viscosity figure was not, therefore, unexpected.

It is further regretted that no physical measurements were possible in the case of B₈, the first sample of blood from case 7, another patient who gave a history of suppression of sweating for three days. The second sample, B₉, from this patient showed high viscosity and low surface tension. It may be mentioned here that considerable difficulty was experienced in precipitating the proteins from the blood sample B₈ by the usual Folin's phosphotungstate method and a double strength had to be used to get a clear filtrate. We consider that this sample would have supported our conclusions if physical measurements had been possible.

Regarding the specific conductivity, an inverse relationship with the severity of the disease is apparent in case 10, a fatal case, which had a rather poor specific conductivity.

Further work on the physical properties is in progress, and a detailed discussion on these will be attempted at a later date.

CLINICAL DATA.

A short summary of the case notes received from hospitals is included as *Appendix B* of this communication.

In 50 per cent of the cases (cases 1, 2, 7, 9, and 10) reflexes had been absent during the period of attack. Willcox (1930-31) considered the disappearance of knee jerks as a significant sign in 'effects of heat' cases and noted that in severe cases its return was delayed for several weeks. Three cases of our series (cases 1, 9, and 10) which showed absence of reflexes ended fatally. Willcox (*loc. cit.*) also suggested estimations of creatinine and indican. Creatinine figures have already been discussed; in none of our urine samples was the presence of indican detected.

Certain diseases, such as typhus, sandfly fever, malaria, focal sepsis, etc., are said to upset the organism's capacity for maintaining the thermal equilibrium with the result that they are more liable to the 'effects of heat'. In our series, case 2 was diagnosed staphylococcal septicaemia from a bad tooth—heat stroke supervening; case 7 had an attack of sandfly fever about 10 days before the onset of 'effects of heat' and case 9 had a benign tertian malarial infection preceding the heat stroke attack.

In almost all our cases mental irritability was an important feature. Cramps and spasmodic contractions of muscles were noted only in three cases, the most marked being in case 10, which not only had a very low blood-chloride figure, but showed definite signs of kidney damage.

Recently Kuo *et al.* (1935) have experimented on human volunteers to determine the relationship between the suppression of sweat and the development of heat stroke. They found that the onset of stupor produced sudden suppression of sweating. Our case notes show that the suppression of sweating was accompanied by such symptoms as mental dullness, extreme restlessness, stuporose condition, and unconsciousness.

These authors concluded from their experiments that suppression of sweating did not play any ætiological rôle in the production of heat stroke but was simply an accompaniment in severe cases. Their conclusion is not understood in view of the fact that with a rise in temperature of the surrounding air to about blood heat, conduction and radiation, the two efficient means of heat loss in temperate climates, become more or less inactive, the only effective factor under such circumstances being evaporation of sweat (Marsh, 1930-31).

Whatever may be their findings in experimental heat stroke, it certainly does not seem to hold good in the case of actual patients. In our series, cases 3 and 7 suffered from suppression of sweating for 18 to 72 hours prior to the development of hyperpyrexia. We have no doubt that this was a contributory factor in causing heat stroke in these cases. A break-down in adaptive equilibrium between body and environment should have resulted from suppression of sweating, the important means of heat loss in the tropics; and as Lee (1935) has pointed out, one of the results of such a break-down among others is hyperpyrexia.

METEOROLOGICAL CONDITIONS.

Charts showing the weekly meteorological conditions in Allahabad, Jhansi, Lahore, Multan, and Sialkot, the stations furnishing 'effects of heat' cases, are incorporated as *Appendix C*. Meteorological data for Ferozepur, another station with one case, are not available.

As has already been mentioned the evaporation of sweat is the most efficient means of heat loss in the tropics, particularly after the temperature has exceeded the blood-heat level. It is needless to add that, with a high atmospheric temperature, high humidity and low wind velocity, the conditions are most unsuitable for physiological adaptation by the human organism. A break-down of equilibrium between the body and environment should be a natural corollary under such circumstances.

Taylor (1919) in his extensive study on meteorological conditions in heat stroke has shown that the maximum number of cases occurred when the combined figures of wet and dry bulb readings reached the highest level. He concluded that the minimum level for the occurrence of heat stroke amongst his cases was a dry bulb temperature of 110°F. with a wet bulb reading of 85°F. or over. There was a greater likelihood of the occurrence of heat stroke in men with no previous experience of the tropics.

In our series out of a total number of 8 British cases (cases 1 to 8) 25 per cent had not experienced any tropical summer before and 25 per cent were in India for the second summer. The remainder, apart from one man (case 2) who was probably born in India and had put in the whole period of his service in India, had been in the tropics for from 2½ to 5 years.

Of the two cases that occurred at Lahore, the original station of one (case 5) was Multan, another hot station in the Punjab. A study of the meteorological conditions at Multan for the week before his attack (17th to 24th July, 1936) shows a steady rise in temperature with a wet bulb reading of about 85°F., a humidity of 80 per cent or over, and a rather low wind velocity. This case must have reached the verge of adaptive equilibrium at Multan. Even at Lahore on the day of his arrival (25th July, 1936) fairly high temperature and humidity figures with a low wind velocity are apparent from the chart.

The second case at Lahore occurred under conditions similar to the above. He was a cook. With a high humidity of about 100 per cent during a period of over three weeks prior to admission and with other contributory factors it is not surprising that a break-down occurred in this case.

The case from Multan proper was at a time when the temperature was steadily rising and had crossed the 110°F. limit with a wet bulb reading of 82°F. to 84°F. during the previous week, although the humidity was falling appreciably. It is, however, interesting to note that the wind velocity suddenly started to diminish from 5 miles per hour during the previous week to 3 miles per hour. It appears to us likely that the sudden fall in wind velocity may have been the precipitating factor, other conditions being already against the man.

There were three cases from Sialkot: the first case on 28th May, 1936, was one of heat exhaustion. The second case occurred during the last week of July in a person who had been in the tropics for only four months. It would appear from the graphs that for three weeks continuously, till about 12 days prior to the attack, the humidity was 100 per cent. On the day of attack the humidity was 88 per cent. This with a temperature just above the blood heat, coupled with 4 hours of exposure a day while doing fatigue duty as a defaulter, in a man who had not had time enough to adequately adapt himself to tropical conditions during a period of 4 months, would in our opinion be enough to upset the equilibrium, particularly in view of his having suffered from sandfly fever about ten days before. The third case at Sialkot was a man who had 2½ years in India to adapt himself. The weather conditions then prevalent do not appear to have been too severe. Perhaps his long exposure to the sun as a defaulter, and continued exposure on the afternoon of 28th August, 1936, after his having fainted while working during the same morning, acted as the exciting factor.

As regards the case from Jhansi the question of adaptation does not arise since his whole service of 28 years had been spent in India. None of the conditions discussed above seems to explain fully the incidence of this case. He suffered from a focal sepsis and developed hyperpyrexia while in hospital. It is likely that the septic condition was the exciting factor in this case.

The solitary case at Ferozepur, for which no meteorological data are available, was a man with only four months' residence in India and it is probable that his failure of adaptation to the tropical conditions was responsible for exciting the attack.

The two cases at Allahabad were Indians, and discussion on them at this stage is not attempted.

SUMMARY.

1. The biochemical findings and physical conditions in ten persons suffering from 'effects of heat' have been studied.

2. A tendency towards retention of nitrogenous constituents in the blood has been observed. This along with the increased blood creatinine and inorganic phosphates, and low blood calcium figures suggests the possibility of kidney insufficiency in severe cases of heat stroke.

The presence of albumin, casts, etc., in the urine of such cases supports the suggestion.

3. The low chloride findings in blood and urine reported by previous workers have been confirmed.

4. An increase in blood-lactic acid and other anions has been observed.

5. It is suggested that a possible contributory factor to hypochloræmia in heat-stroke cases is the high anion concentration in blood, in the light of the 'Donnan equilibrium'.

6. A preliminary discussion on the physical states of the blood and the clinical condition in 'effects of heat' cases is given.

7. A short study has been made of meteorological conditions and their correlation with the occurrence of cases.

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TABLE I.

Analytical methods employed in biochemical estimations.

Specimen.	Nature of estimation.	References to the methods adopted.
<i>A. Blood samples.</i>		
1. Oxalated blood ..	N.P.N., urea, uric acid, and creatinine.	Folin (1934).
	Chlorides.	Whitehorn (1921).
	Lactic acid.	Boyland (1928).
2. Citrated blood ..	Total bases.	Benzidine method, Fiske (1922).
	Potassium.	Cobaltinitrite method, Clausen (1918) and Kramer and Tisdall (1921).
	Inorganic phosphates.	Briggs (1922).
3. Serum ..	Calcium.	Beaumont and Dodds (1934).
	Cholesterol.	Myers and Wardell (1918).
	Protein.	Wu and Ling's colorimetric method modified by Greenberg (1929).
<i>B. Urine samples.</i>		
	Total nitrogen, urea, uric acid, creatinine, ammonia, sulphates, chlorides, total bases, calcium, magnesium, and potassium.	Folin (1934).
	Inorganic phosphates.	Briggs (1922).

TABLE II-A.

Nitrogenous constituents in blood.

Case number.	Index number. Date of collection of sample.	UREA.	URIC ACID.	CREATININE.	AMINO ACID AND UNDETER- MINED NITROGEN.	TOTAL NON-PROTEIN NITROGEN.
		Mg. per 100 c.c. 'N', mg. per 100 c.c.	Mg. per 100 c.c. 'N', mg. per 100 c.c.	Mg. per 100 c.c. 'N', mg. per 100 c.c.	'N', mg. per 100 c.c.	'N', mg. per 100 c.c.
1 {	B ₁ 21-5-36	153.04 71.32	6.60 2.20	4.215 1.56	} 18.67	93.75
2 {	B ₂ 16-6-36	70.125 32.68	3.904 1.301	1.45 0.565	} 11.204	45.75
	B ₄ 27-6-36	78.75 36.70	3.77 1.26	1.456 0.539	} 5.351	43.85
3 {	B ₃ 19-6-36	97.39 45.38	2.88 0.96	1.935 0.716	} 8.494	55.55
4 {	B ₅ 25-7-36	62.41 29.08	2.964 0.988	1.485 0.549	} 27.883	58.50
5 {	B ₆ 26-7-36	119.3 55.62	3.296 1.098	2.08 0.77	} 24.212	81.70
6 {	B ₇ 5-8-36	89.25 41.59	2.208 0.736	1.604 0.593	} 12.631	55.55

TABLE II-A—concl'd.

Case number.	Index number. Date of collection of sample.	UREA.	URIC ACID.	CREATININE.	AMINO ACID AND UNDETERMINED NITROGEN.	TOTAL NON-PROTEIN NITROGEN.
		Mg. per 100 c.c. 'N', mg. per 100 c.c.	Mg. per 100 c.c. 'N', mg. per 100 c.c.	Mg. per 100 c.c. 'N', mg. per 100 c.c.	'N', mg. per 100 c.c.	'N', mg. per 100 c.c.
7	B ₈ 4-8-36	132.85 61.90	3.56 1.18	2.145 0.793	35.807	100.30
	B ₉ 8-8-36	189.0 88.07	3.52 1.17	2.385 0.882	21.078	111.20
	B ₁₂ 15-9-36	70.85 33.02	3.20 1.06	1.464 0.542	7.048	41.67
8	B ₁₀ 29-8-36	130.7 60.81	2.192 0.730	2.55 0.943	61.617	124.2
	B ₁₁ 8-9-36	80.53 37.53	2.264 0.754	1.45 0.565	12.051	50.9
9	CB ₁ 21-5-36	112.9 52.61	5.80 1.93	1.818 0.673	8.487	63.7
10	CB ₂ 29-5-36	53.04 24.72	6.60 2.20	1.67 0.618	39.832	67.37
	CB ₃ 30-5-36	76.87 35.81	17.72 5.90	2.80 1.036	97.254	140.0

N.B.—The figures in the table correspond to the order in the headings of the respective columns. 'N' = nitrogen.

TABLE II-B.

Nitrogenous constituents in urine.

Case number.	Index number. Date of collection of sample. Total quantity in 24 hours in c.c.	UREA.	URIC ACID.	CREATININE.	AMMONIA.	AMINO ACID AND UNDETERMINED NITROGEN.	TOTAL NITROGEN.
		Mg. per 100 c.c. 'N', mg. per 100 c.c. Total urea excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total uric acid excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total creatinine excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total ammonia excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.
1	No urine samples.				
2	UB ₂ I 16/17-6-36 850	444.0 206.9 3.774	29.2 9.7 0.248	Trace	152.8 125.75 1.299	103.15 0.877	445.5 3.787
	UB ₂ II 18/19-6-36 875	303.0 141.3 2.651	8.16 2.72 0.071	Trace	36.21 29.80 0.317	6.18 0.054	180.0 1.575
	UB ₂ III 21/22-6-36 900	263.6 122.84 2.372	17.65 5.88 0.159	Trace	143.87 118.40 1.295	86.13 0.775	333.25 2.999
	UB ₂ IV 24/25-6-36 825	179.9 83.93 1.484	29.29 9.76 0.242	Trace	425.9 350.51 3.514	55.80 0.460	500.0 4.125
	UB ₂ V 10/11-7-36 850	354.2 165.06 3.010	42.84 14.28 0.364	95.2 35.22 0.809	418.7 344.59 3.558	280.85 2.387	840.0 7.140
3	UB ₃ I 18/19-6-36 855	953.0 444.10 8.148	34.087 11.362 0.291	Trace	115.6 95.14 0.988	102.898 0.880	653.5 5.587

TABLE II-B—contd.

Index number.	UREA.	URIC ACID.	CREATININE.	AMMONIA.	AMINO ACID AND UNDETERMINED NITROGEN.	TOTAL NITROGEN.
Date of collection of sample Total quantity in 24 hours in c.c.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total urea excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total uric acid excre- tion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total creatinine excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total ammonia excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.
UB ₁ 24/25-7-36 1,500	970.95 452.47 14.564	42.34 14.11 0.635	84.0 31.08 1.260	290.1 238.75 4.352	197.59 2.964	934.00 14.01
UB ₁ 25/26-7-36 1,280	502.7 234.26 6.435	44.44 14.81 0.569	86.2 31.89 1.104	176.6 145.34 2.260	314.30 4.043	740.60 9.480
UB ₁ 5/6-8-36 5,020	369.67 172.27 18.557	17.64 5.88 0.885	16.46 6.09 0.826	26.98 22.20 1.354	51.86 2.603	258.00 12.951
UB ₁ 4/5-8-36 3,185	287.58 134.01 9.159	3.128 1.042 0.100	20.4 7.55 0.650	159.67 131.41 5.087	57.738 1.839	331.75 10.566
UB ₂ 8/9-8-36 2,010	279.0 130.01 5.608	27.02 9.00 0.543	53.45 19.78 1.074	379.34 312.20 7.625	53.51 1.075	524.50 10.543
UB ₃ 15/16-9-36 3,010	214.71 100.05 6.463	17.64 5.88 0.531	50.65 18.74 1.524	52.57 43.26 1.582	138.07 4.156	306.00 9.210

TABLE II-B—concl'd.

Case number.	Index number. Date of collection of sample. Total quantity in 24 hours in c.c.	UREA.	URIC ACID.	CREATININE.	AMMONIA.	AMINO ACID AND UNDETERMINED NITROGEN.	TOTAL NITROGEN.
		Mg. per 100 c.c. 'N', mg. per 100 c.c. Total urea excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total uric acid excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total creatinine excretion in 24 hours in g.	Mg. per 100 c.c. 'N', mg. per 100 c.c. Total ammonia excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.	'N', mg. per 100 c.c. Total excretion in 24 hours in g.
8	UB ₁₀ I 28/29-8-36 3,500	414.64 193.22 14.512	14.8 4.9 0.518	93.0 34.41 3.255	95.8 78.84 3.353	12.33 0.432	323.7 11.329
9	No urine samples.				
10	UCB ₁₀ I 30-5-36 *	722.14 336.51	23.24 7.74	109.9 40.66	121.4 99.91	219.18	704

* A catheter specimen, only 80 c.c. drawn. Had 0.389 per cent albumin, casts, blood, etc.

N.B.—The figures in the tables correspond to the order in the headings of the respective columns.
'N' = nitrogen.

TABLE III-A.
Acid and base radicals in blood.

Case number.	Index number. Date of collection.	ANIONS.					CATIONS.				
		Chlorides. As NaCl, mg. per 100 c.c. ME. of blood.	Phosphates. As inorganic, 'P', mg. per 100 c.c. mM. in blood. ME. of blood. [ME.=mM×1.8]	Proteins in serum. Total per 100 c.c. of serum in g. Total per 1,000 c.c. of serum in g.=P. Base combining power. ME. of serum. (B protein = 0.242 × P.)	Lactic acid. Mg. per 100 c.c. ME. of blood.	Undeter- mined radicals including sulphates. ME. of blood.	Total bases. ME. of blood.	Calcium. Mg. per 100 c.c. ME. of blood.	Potassium. Mg. per 100 c.c. ME. of blood.	Magnesium (normal figures). Mg. per 100 c.c. ME. of blood.	Sodium and other undeter- mined cations (calculated). Mg. per 100 c.c. ME. of blood.
1	B ₁ 21-5-36	314.0 .. 53.6	14.2 4.6 8.82	No serum 204.0	7.55 .. 3.75	144.2 .. 36.97	3.6 .. 2.96	368.7 .. 160.32
2	B ₂ 16-6-36	380.0 .. 64.9	14.7 4.7 8.46	7.5143 75.143 18.25	50.4 .. 5.6 98.79 196.0	10.80 .. 5.4	230.84 .. 59.29	3.6 .. 2.96	295.205 .. 128.35
3	B ₃ 27-6-36	400.0 .. 68.3	24.76 7.9 14.22	7.0660 70.660 17.17	31.8 .. 3.5 54.08 157.27	11.2 .. 5.6	180.0 .. 46.15	3.89* .. 3.19	235.36 .. 102.33

TABLE III—A—concl.

Case number.	Index number. Date of collection.	ANIONS.					CATIONS.				
		Chlorides. As NaCl, mg. per 100 c.c. ME. of blood.	Phosphates. As inorganic, 'P', mg. per 100 c.c. mM. in blood. ME. of blood. [ME. = mM × 1.8]	Proteins in serum. Total per 100 c.c. of serum in g. Total per 1,000 c.c. of serum in g. = P. Base combining power. ME. of serum. (B protein = 0.243 × P.)	Lactic acid. Mg. per 100 c.c. ME. of blood.	Under- mined radicals including sulphates. ME. of blood.	Total bases. ME. of blood.	Calcium. Mg. per 100 c.c. ME. of blood.	Potassium. Mg. per 100 c.c. ME. of blood.	Magnesium (normal figures). Mg. per 100 c.c. ME. of blood.	Sodium and other under- mined cations (calculated). Mg. per 100 c.c. ME. of blood.
3	B ₁ 19-6-36	415.0 70.9	29.64 9.6 17.82	No serum	43.2 .. 4.8 178.26 No serum	132.3 .. 33.9
4	B ₂ 25-7-36	380.0 .. 64.9	24.6 7.9 14.22	8.7207 87.207 21.19	64.2 .. 7.1 50.99 158.4	11.43 .. 5.72	146.8 .. 37.64	3.6 .. 2.96	257.8 .. 112.08
5	B ₀ 26-7-36	360.0 .. 61.5	19.52 6.3 11.34	7.8126 78.126 18.98	52.1 .. 5.8 75.68 173.3	12.63 .. 6.32	124.0 .. 31.79	3.6 .. 2.96	304.13 .. 132.23
6	B ₇ 5-8-36	380.0 .. 64.9	19.14 6.2 11.12	6.2890 62.890 15.28	35.1 .. 3.9 77.56 172.8	11.79 .. 5.89	114.6 .. 29.38	3.6 .. 2.96	309.51 .. 134.57

7	B ₂ 4-8-36	360-0 .. 61.5	29-100 9.4 16.92	8-3148 83-148 20-20	40-50 .. 4.5 No blood ..	11.2 .. 5.6	114.0 .. 29.23
		380-0 .. 64.9	27-76 8.9 16.02	7-2304 72-304 17-56	38-18 .. 4.2	.. 77.19 179.87 ..	11.4 .. 5.7	99.8 .. 25.59	3.6 2.96 ..	334.97 145.62 ..
	B ₂ 15-9-36	420-0 .. 71.7	20-40 6.6 11.88	7-3900 73-900 17-95	Trace 56.92 158.45 ..	12.24 .. 6.12	110.2 .. 28.26	3.6 2.96 ..	278.87 121.25 ..
8	B ₁₀ 29-8-36	400-0 .. 68.3	20-60 6.6 11.88	6-7030 67-030 16-28	46-575 .. 5.2	.. 86.64 188.3 ..	12.58 .. 6.29	114.0 .. 29.23	3.6 2.96 ..	344.50 149.82 ..
		440-0 .. 75.2	No blood	6-6450 66-450 16-16	No blood No blood ..	12.09 .. 6.04	No blood
	B ₁₁ 8-9-36	300-0 .. 61.5	10-80 3.30 6.30	No serum 220.0 ..	7.25 .. 3.62	219.8 .. 56.36	3.6 2.96 ..	301.24 157.06 ..
9	CB ₂ 21-5-36	380-0 .. 64.9	10-50 3.4 6.12	No serum No blood ..	9.80 .. 4.9	102.56 .. 26.30	3.6 2.96
		340-0 .. 58.1	15-18 4.9 8.82	No serum	42-48 .. 4.7	6.80 .. 3.45	170.16 .. 43.63	3.6 2.96 ..	432.31 187.96 ..
	CB ₂ 30-5-36										
10	CB ₂ 29-5-36										

* Actual figure of magnesium.
N.B.—The figures in the table correspond to the order in the headings of the respective columns.
 mM = millimolar concentration.
 ME. = Milli-equivalents per 1,000 c.c.

TABLE III-B.
Acid and base radicals in urine.

Case number.	ANIONS.				CATIONS.			
	Chlorides. As NaCl, mg. per 100 c.c. Excretion in 24 hours in g. ME. of urine.	Sulphates. As H ₂ SO ₄ , mg. per 100 c.c. Excretion in 24 hours in g. mM. in urine.	Phosphates inorganic. As 'P', mg. per 100 c.c. As P ₂ O ₅ , mg. per 100 c.c. Excretion in 24 hours as P ₂ O ₅ in g. mM. in urine.	Total bases. ME. of urine.	Calcium. As 'Ca', mg. per 100 c.c. As 'CaO', mg. per 100 c.c. Excretion in 24 hours as 'CaO', in g. ME. of urine.	Magnesium. As 'Mg', mg. per 100 c.c. As 'MgO', mg. per 100 c.c. Excretion in 24 hours as 'MgO', in g. ME. of urine.	Potassium. Mg. per 100 c.c. Excretion in 24 hours in g. ME. of urine.	Sodium (calculated). Mg. per 100 c.c. (calculated). Excretion in 24 hours in g. ME. of urine.
1	No urine samples.			
UB ₁ I 1002 850 17-6-36	16 .. 0.136 2.7	132.5 .. 1.126 27.04	19.9 45.57 0.387 6.4 54.99	19.59 27.4 0.233 9.79	4.194 6.95 0.059 3.4	34.4 .. 0.292 8.82	75.85 .. 0.638 32.98
UB ₂ II 1003 875 19-6-36	12 .. 0.105 2.05	85.26 .. 0.746 17.4	11.43 26.17 0.229 3.7 36.7	17.40 24.36 0.213 8.70	3.330 5.52 0.048 2.7	24.8 .. 0.217 6.36	43.56 .. 0.381 18.94
UB ₃ III 1004 900 22-6-36	25 .. 0.225 4.2	114.27 .. 1.023 23.32	25.6 58.62 0.528 8.2 61.3	23.46 33.04 0.207 11.7	1.4706 2.44 0.022 1.2	39.00 .. 0.351 10.0	88.32 .. 0.795 38.4

3	UP ₂ IV 1010 825 25-6-36	490 .. 4-043 83.7	259.19 .. 2-138 52.88	41.66 95.40 0.787 13.4 158.3	24.35 34.09 0.281 12.17	4.3338 7.185 0.060 3.5	88.1 .. 0.727 22.59	276.09 .. 2.278 120.04
	UP ₂ V 1012 350 11-7-36	560 .. 4-760 96.7	208.5 .. 2.282 54.8	39.38 90.18 0.767 12.7 186.8	51.26 71.76 0.61 25.6	Trace	153.1 .. 1.301 39.25	280.455 .. 2.384 121.95
4	UB ₂ I 1008 855 19-6-36	60 .. 0.513 10.2	160.72 .. 1.374 32.8	28.56 65.40 0.559 9.2 59.8	14.13 19.78 0.170 7.06	4.2636 7.07 0.060 3.6	58.5 .. 0.500 15.51	77.35 .. 0.651 33.63
	UB ₂ I 1009 1500 25-7-36	32 .. 0.480 5.4	121.28 .. 1.819 24.75	20.62 47.21 0.708 6.6 81.4	2.85 3.99 0.060 1.42	2.124 3.52 0.053 1.7	64.81 .. 0.972 16.61	141.84 .. 2.128 61.67
5	UB ₂ I 1006 1280 26-7-36	88.0 .. 1.126 15.04	73.74 .. 0.944 15.05	25.92 58.67 0.750 8.3 103.9	4.33 6.05 0.078 2.16	2.760 4.58 0.059 2.2	186.0 .. 2.380 47.69	119.255 .. 1.526 51.85
	UB ₂ I 1004 5920 6-8-36	15 .. 0.753 2.5	104.5 .. 5.246 21.33	21.85 50.04 2.512 7.05 43.3	5.75 8.05 0.404 2.87	2.478 4.11 0.206 2.04	26.3 .. 1.320 6.74	72.195 .. 3.654 31.65

TABLE III-B—*concd.*

Index number. Specific gravity. Total quantity in 24 hours in c.c. Date of collection.	ANIONS.			Total bases. ME. of urine.	CATIONS.			
	Chlorides. As NaCl, mg. per 100 c.c. Excretion in 24 hours in g. ME. of urine.	Sulphates. As H ₂ SO ₄ , mg. per 100 c.c. Excretion in 24 hours in g. mM. in urine.	Phosphates inorganic. As 'P', mg. per 100 c.c. As P ₂ O ₅ , mg. per 100 c.c. Excretion in 24 hours as P ₂ O ₅ in g. mM. in urine.		Calcium. As 'Ca', mg. per 100 c.c. As 'CaO', mg. per 100 c.c. Excretion in 24 hours as 'CaO' in g. ME. of urine.	Magnesium. As 'Mg', mg. per 100 c.c. As 'MgO', mg. per 100 c.c. Excretion in 24 hours as 'MgO' in g. ME. of urine.	Potassium. Mg. per 100 c.c. Excretion in 24 hours in g. ME. of urine.	Sodium (calculated). Mg. per 100 c.c. (calculated). Excretion in 24 hours in g. ME. of urine.
UB ₁ I 1003 3185 5-8-36	84 .. 2·675 14·3	142·19 .. 4·529 29·02	16·58 37·97 1·209 5·3 54·7	34·22 47·91 1·53 17·11	Trace	64·2 .. 2·045 16·46	48·60 .. 1·550 21·13
UB ₂ II 1005 2010 9-8-36	263·0 .. 5·29 44·9	90·39 .. 1·817 18·45	13·46 30·82 0·619 4·3 107·5	17·75 24·85 0·50 8·9	Trace	173·2 .. 3·481 44·41	124·637 .. 2·505 54·19
UB ₃ III 1004 3020 16-9-36	198 .. 5·960 33·84	80·80 .. 2·432 16·49	17·77 40·69 1·225 5·7 70·4	14·19 19·87 0·60 7·09	1·608 2·67 0·080 1·3	41·7 .. 1·255 10·69	118·036 .. 3·553 51·32

Case number.

7

Case number.

8	UB ₂ I 1004 3500 29-8-36	145.0	84.2	16.2	..	4.54 6.35 0.22 2.27	Trace	85.5	94.32
		..	2.947	37.10	2.982	..
		5.075	17.18	1.299	65.2	21.92	41.01
9	..	No urine sample.				
	
	
10	UCF ₂ I * 30-5-36	60	444.4	4.79	..	9.54 13.36 4.8
		..	90.69	10.97
		10.2	..	1.5

* Only one catheter sample received.
N.B.—The figures in the tables correspond to the order in the headings of the respective columns.
 mll. = millimolar concentration.
 ME. = milli-equivalents per 1,000 c.c.

TABLE IV.

Physical data of blood samples with figures of total protein and cholesterol estimation.

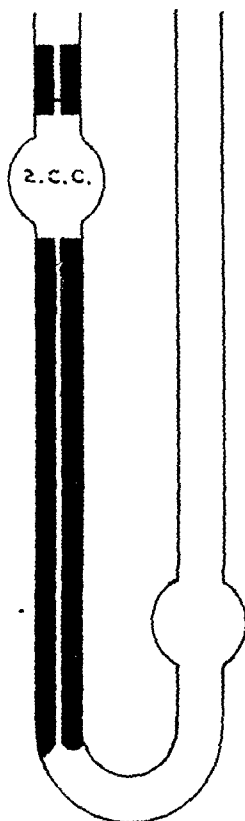
Case number.	Index number of samples.	SPECIFIC GRAVITY OF BLOOD.		VISCOSITY OF BLOOD.		CONDUCTIVITY OF BLOOD.		SURFACE TENSION OF BLOOD, DYNE/CM.					Total cholesterol, mg. per 100 c.c. of serum.	Total proteins, grammes per hundred c.c. of serum.
		Specific gravity.	Atmospheric temperature, °C.	Relative viscosity.	Atmospheric temperature, °C.	Specific conductivity $\times 10^3$.	Atmospheric temperature, °C.	At atmospheric pressure.	At minus 40 mm. water pressure.	At minus 60 mm. water pressure.	Atmospheric temperature, °C.	Surface tension of water at the same temperature.		
2	{ B ₂ B ₄	1054	33.5	4.967	35	10.860	35	61.62	60.04	58.92	35	70.30	200.0	7.5143
		1053	29	4.870	30.5	10.291	30.5	64.31	62.53	60.63	31	70.89	148.0	7.0660
3	B ₃	1057	32	9.510	32	7.136	32	58.22	56.76	55.93	32	70.74	..	No serum available.
		1058	28	6.649	28	9.583	28	60.61	59.64	59.18	28.5	71.26	109.9	8.7207
5	B ₅	1057	28	8.035	28	8.726	28	53.95	52.91	52.19	29.5	71.11	217.4	7.8126
		1058	28	4.593	29.5	8.654	28	61.5	60.86	60.57	29	71.19	77.11	6.2890
7	{ B ₉ B ₁₂	1058	31	5.183	32	8.818	32	56.24	53.60	52.85	32	70.74	138.0	7.2304
		1057	30	5.218	28.5	52.07	51.60	51.13	28.5	71.26	147.8	7.390
8	B ₁₀	1057	30	8.672	30.5	10.450	30	60.73	59.46	58.78	30.5	70.96	83.3	6.703
		1055	35.5	6.449	36	5.611	36
10	{ CB ₂ CB ₃	1053	35	6.992	38	7.013	38
		1053	35	6.992	38	7.013	38

APPENDIX A.

DETAILS OF TECHNIQUE FOLLOWED FOR THE PHYSICAL MEASUREMENTS.

PHYSICAL data of blood samples were obtained from citrated blood.

1. *Electrical conductivity.*—It is well known that the electrical conductivity of a solution depends on the presence of the ions obtained from the electrolytes in solution. A careful investigation of the physical properties, therefore, gives an insight as to the increase or decrease of such conducting substances in blood. The method adopted for the determination of electrical conductivity was the Wheatstone bridge with the introduction of an alternating current from a toy induction coil in place of direct current from a battery. A telephone was used to find the null point.



TEXT-FIG. 1.

The blood was placed in a small conductivity cell and its resistance between two plates of platinum coated with platinum-black was noted. The cell-constant was obtained by using a standard solution of KCl, an electrolyte of known conductivity. The specific conductivity figures for the blood samples were then obtained by calculation.

2. *Viscosity*.—The importance of this physical property for colloidal systems has been known from the time of Graham (1864). The variations in viscosity of protein solutions at different hydrogen ion concentrations have been observed by Loeb (1922) and other investigators in colloid chemistry.

For viscosity measurements a micro viscosimeter of Ostwald's type was used throughout (Text-fig. 1) and the time of flow of 2 c.c. citrated blood was noted by a stop-watch; the average of three readings was finally recorded. Measurements were taken at room temperature.

The time of flow of 2 c.c. water at the same temperature was also recorded and viscosity of the samples of blood was calculated by applying the formula :—

$$\frac{n_s}{n_w} = \frac{t_s d_s}{t_w d_w}$$

where n_s , t_s , and d_s are viscosity, time of flow and density respectively of the sample of blood and n_w , t_w , and d_w those of water at the same temperature. The figures for the viscosity of water were taken from Landolt's (1923) tables. From the specific viscosities thus obtained relative viscosities were worked out by taking that of the water, at that particular temperature, as unity.

The usual precautions were adopted of washing the viscosimeter and carefully drying by passing warm air through prior to use.

3. *Surface tension*.—The presence of protein colloids or other bio-colloids in a system alters considerably the surface tension of the dispersing medium. It has, however, been noted that with such colloids different surface tension values are obtained according to the rate at which the drops are allowed to form. Traube's stalagmometer, therefore, has been used for the determination of the surface tension with the following modifications :—

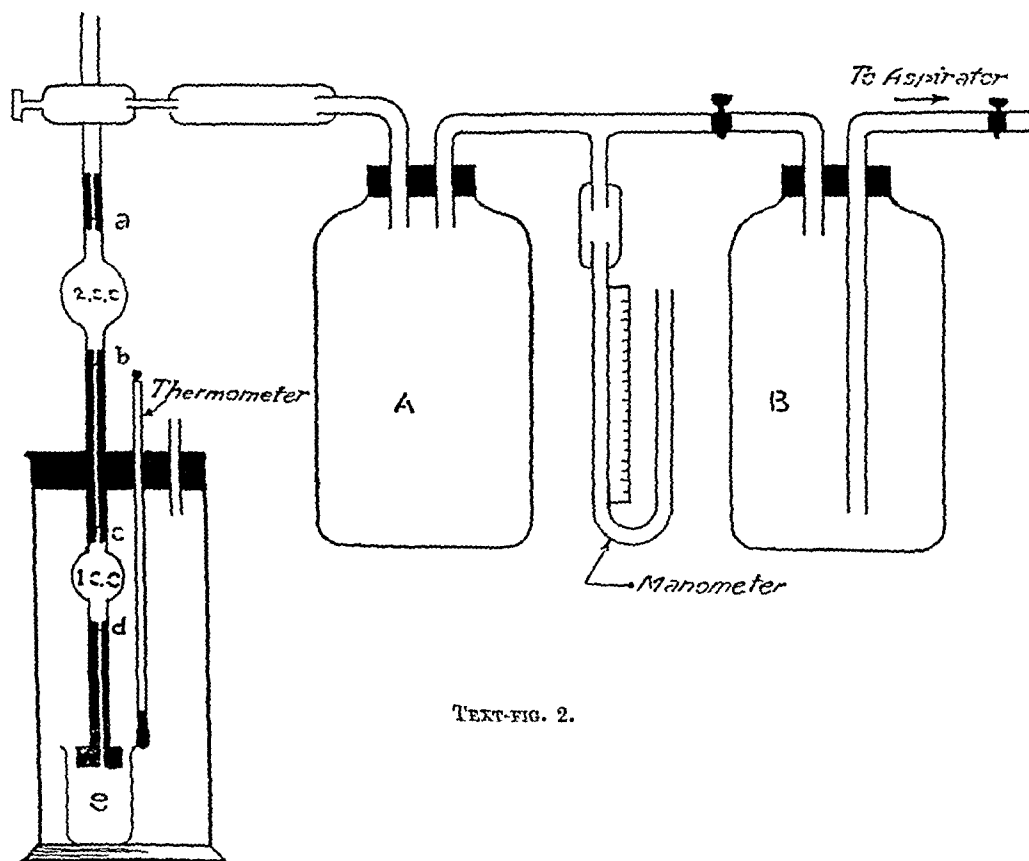
A special stalagmometer pipette suitable for both 1 c.c. and 2 c.c. of blood was used. Measurements were taken at atmospheric pressure, at water pressures of minus 40 and minus 60 mm. For this purpose the apparatus was fitted up as in Text-fig. 2. The stalagmometer was encased in a wide-mouthed cylinder in order to prevent the influence of air currents on the drops. A three-way stop-cock was fixed to the upper end of the stalagmometer pipette which could be manipulated in such a way that the pipette could be connected at will either to the atmospheric pressure or to lower pressures in bottle 'A', the manometer indicating the actual pressure. Bottle 'B' was filled with water and connected at one end to the aspirator. Aspiration of water from bottle 'B' gave a negative pressure in the system. By careful manipulation maintenance of water pressure of minus 40 mm. or minus 60 mm. was readily obtained.

The stalagmometer was filled to mark 'a' with blood and the number of drops formed at atmospheric pressure in 2 c.c. of blood in the course of a fall in level from 'a' to 'b' was counted and the time taken for this was measured by a stop-watch. One c.c. measurements were taken by the fall in level from 'c' to 'd'. The blood drops were collected in a small flat-bottomed tube 'e' and sucked again into the stalagmometer pipette for the next series of measurements at pressures of minus 40 mm. and minus 60 mm.

Similar measurements were taken for water with the same stalagmometer pipette as was used for blood samples, and the number of drops for 2 c.c. and 1 c.c. quantities at 28°C. at various pressures was noted. From these values temperature corrections for the number of water-drops at the temperatures of blood samples were calculated by the formula :—

$$n_2 = \frac{r_1 n_1 \rho_2}{r_2 \rho_1}$$

where r_1 , n_1 , and ρ_1 are surface tension, number of drops, and density of water at 28°C.; and r_2 , n_2 , and ρ_2 are those at the required temperature.



TEXT-FIG. 2.

Figures for surface tension and density of water were obtained by interpolating the data available in Landolt's (*loc. cit.*) tables.

Thus, knowing the number of drops that 2 c.c. and 1 c.c. of water would give at the temperatures of the blood samples, the surface tensions of blood at the

atmospheric, minus 40 and minus 60 mm., pressures respectively were finally calculated by adopting the following formula :—

$$r_b = \frac{r_w n_w p_b}{n_b p_w}$$

where r_w , n_w , and p_w are surface tension, number of drops, and density of water, and r_b , n_b , and p_b those of the blood samples at the same temperature.

All data except r_b being known it was an easy matter to arrive at the surface tension figures in Dyne/cm. (*see* Table IV).

It is known that the number of drops formed out of the same quantity of a fluid in a stalagmometer is inversely proportional to the surface tension and the effective atmospheric pressure. A similar state of affairs was found in our series of observations; the same sample of blood gave a larger number of drops and consequently lower surface tension figures with readings at minus 40 mm. and minus 60 mm. as compared with the result at atmospheric pressures (*see* Table IV).

The usefulness of surface tension data at different pressures will form the subject of a separate study at a later date.

4. *Density*.—In the course of the determination of viscosity and surface tension it has been necessary to determine the density of blood. Density bottles, 2 c.c. or 4 c.c., were used to obtain relative density figures. These results are also recorded (*see* Table IV).

APPENDIX B.

SUMMARY OF CASE NOTES.

Case 1.—C. B., aged 21 years, total service 3 years, service in India 1 year and 2 months, station Sialkot.

A fatal case of heat exhaustion. Maximum temperature recorded 105·6°F. Had 13½ hours' pyrexia. Originally admitted five days before attack with vague epigastric pain, nausea, and constipation. Special signs and symptoms during attack were abdominal discomfort, nausea, mental dullness, lack of sweating, absence of knee jerks, weak ankle jerks and abdominal reflex, eye reflexes normal. The patient suffered from auricular fibrillation just before death.

For three days prior to admission he was exposed to the sun for about 5½ hours daily. Dress—shirt, slacks, and topee. Had dysentery in April 1936. An abstainer, regimentally employed as a transport driver.

Case 2.—D. F., aged 47, total service in India 28 years, station Jhansi.

A case diagnosed as 'staphylococcal septicaemia from a bad tooth, with heat stroke superimposed'.

The heat-stroke attack occurred 10 days after his admission for backache, frequency of micturition and irregular temperature. Maximum temperature recorded was 106°F. During the period prior to the attack he suffered from irregular temperature with a maximum of 103·4°F. Total duration of pyrexia before, during, and after the attack was 37 days, followed by slow recovery.

Special signs and symptoms noted were cerebral irritation with irregular pupils and sluggish eye reflexes, absence of knee and ankle jerks, and pain in the lumbar region.

Laboratory tests for enteric group negative throughout. Agglutination only present against OXK in 1/50. Apart from the presence of leucocytosis to 9,000 per c.mm. no cytological abnormalities were noted; no malarial parasites detected. W. R. of blood and C.S.F. negative. Urea concentration test normal. Urine showed presence of pus and epithelial cells on many occasions. Blood pressure on the day following the attack was 100/80 and 8 days later was 90/70.

An abstainer, employed mostly in office work with an average of about 2 hours a day outdoor duties. Dress—shorts, shirt and topee.

Case 3.—S. J., aged 27, total service 3 years, service in India 1 year and 5 months, station Multan.

A case of heat stroke ending in complete and uncomplicated rapid recovery. Maximum temperature recorded 106·2°F. Duration of pyrexia 4 days. Blood smear negative for malaria parasites.

Special symptoms noted during the attack were extreme restlessness with hot, dry, and cyanosed skin. Eighteen hours prior to the attack suffered from anorexia, frontal headache, vertigo, restlessness, constipation, and suppressed sweating.

An abstainer and an athlete; was doing range firing 3 hours a day for 3 days prior to the attack. Dress—shorts, shirt, boots, putties, and topee.

Case 4.—N. A., aged 30, total service 5 years, service in India 3 years, station Lahore.

A case of heat-hyperpyrexia ending in rapid recovery. Maximum temperature recorded 108·2°F. (rectal). Duration of pyrexia 3 days.

Special signs and symptoms noted were semiconsciousness at the time of admission with a rectal temperature of 107·1°F., dilated pupils, cyanosed, and stertorous breathing.

Employed approximately 12 hours daily in cook-house. An abstainer and a man of good physique.

Case 5.—B. W., aged 36, total service 21 years, service in India 5 years, station Lahore.

A case of heat stroke ending in recovery. Original station Multan, was attacked while in train on way to hills and evacuated at Lahore. Maximum temperature recorded 105·4°F., duration of pyrexia 8 days. Blood smear negative for malaria parasites.

No special signs and symptoms noted except dry skin.

Usually consumed 6 bottles of beer a day but on the day of attack had only one.

Case 6.—T. T., aged 23 years, total service 1 year and 8 months, service in India 4 months, station Ferozepore.

A case of heat stroke ending in recovery. Maximum temperature recorded 106·6°F., duration of pyrexia 6 days. Blood smears negative for malaria parasites.

Special signs and symptoms noted were headache, constipation, cramps, mental dullness, stuporose condition, hot and dry skin, contracted and inactive pupils, and lack of sweating.

Was employed in military duty with an average of 1½ hours' exposure to the sun daily. Dress—shirt, shorts, boots, putties, topee, and equipment.

Had 1½ pints of beer on the day previous, suffered from headache, malaise, and constipation 2 days prior to attack.

Case 7.—W. R., aged 21, total service 2 years, service in India 4 months, station Sialkot.

A case of heat stroke ending in slow recovery. Maximum temperature recorded 105°F. Hyperpyrexia for a few hours followed by continuous temperature for 26 days and irregular low fever for a further 37 days. Blood smears negative for malaria parasites; cultures negative for 'E' group throughout, widal no rise.

The patient being a defaulter was employed in grooming and weeding. Was exposed for about 4 hours a day. Dress—slacks, vest, and topee. He fell down unconscious at 7 p.m. on the day of attack after weeding for one hour in the sun, and was admitted to the hospital as such.

Special signs and symptoms noted were lack of sweating with hot and dry skin, pupils equal but inactive, absence of reflexes, condition hysterical and delirious.

About 10 days prior to the attack suffered from sandfly fever, and from suppressed sweating 3 days prior to the admission.

Consumed beer occasionally.

Case 8.—N. G., aged 24, total service $4\frac{1}{2}$ years, service in India $2\frac{1}{2}$ years, station Sialkot.

A case of heat stroke ending in recovery. Maximum temperature recorded 106°F . Duration of pyrexia 4 days. Blood smears negative for malaria parasites; cultures and widal negative for 'E' group.

As a defaulter was on fatigue duty and was employed in weeding, digging, and cutting grass. Was exposed to the sun for $4\frac{1}{4}$ hours daily. Dress—shirt and slacks.

At about 10-30 a.m. on the day of attack while cutting grass he had fainted, but was again employed on fatigue duty from 4 p.m. to 5-55 p.m. He was subsequently admitted to hospital at 6 p.m. on the same day with hyperpyrexia.

Special signs and symptoms noted were weakness and aching of arms and legs, cramps in calves and normal reflexes.

Usually consumed 2 bottles of beer a week.

Case 9.—D. D., aged 25 years, a civilian (Indian), station Allahabad.

A fatal case of heat stroke. Was picked up from road side in an unconscious state four days before death. Benign tertian gametocytes were seen in blood smears; but anti-malarial treatment did not relieve. Maximum temperature recorded 108.6°F . (axillary). Duration of hyperpyrexia $8\frac{1}{2}$ hours ending in death.

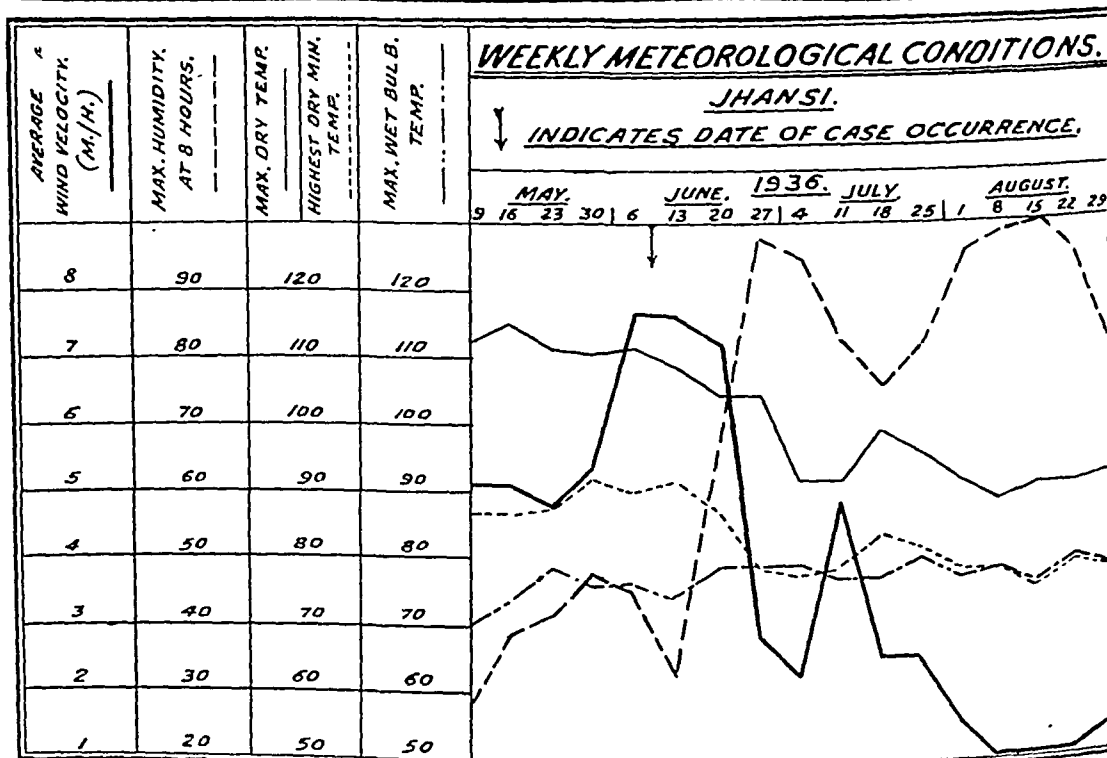
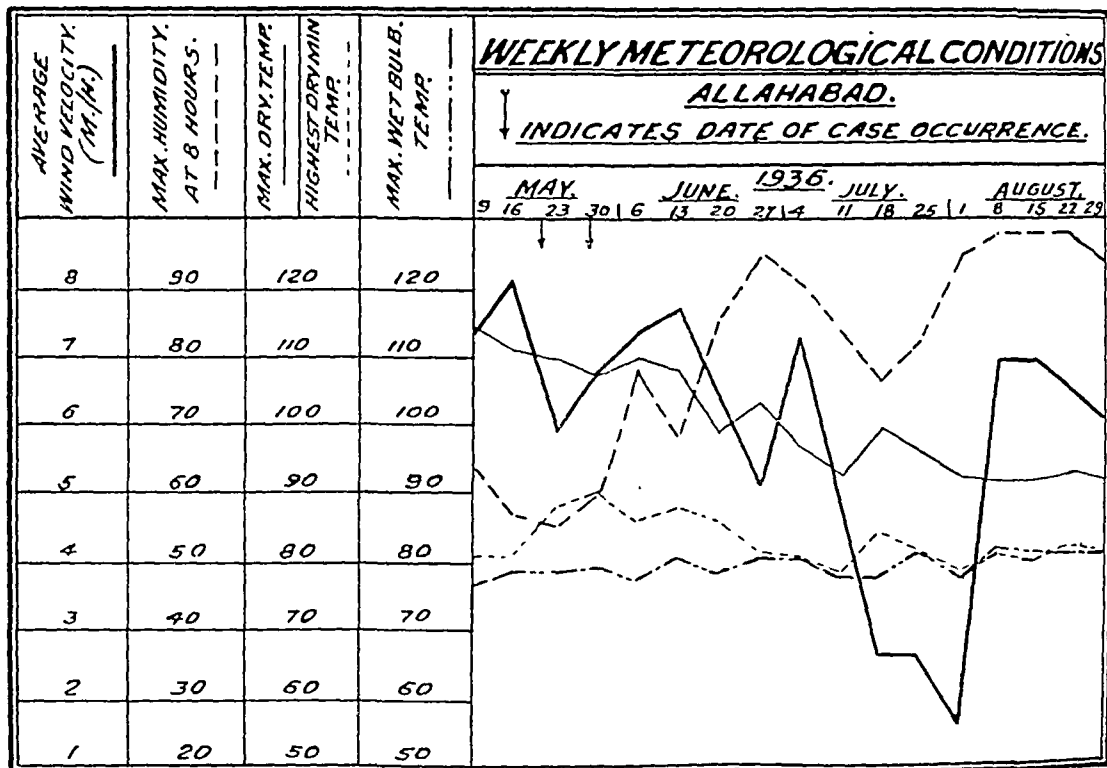
Special signs and symptoms noted were unconsciousness, absence of reflexes, pupils dilated and inactive, subconjunctival hæmorrhages, and stertorous breathing. Blood pressure 2 hours prior to death 90/70.

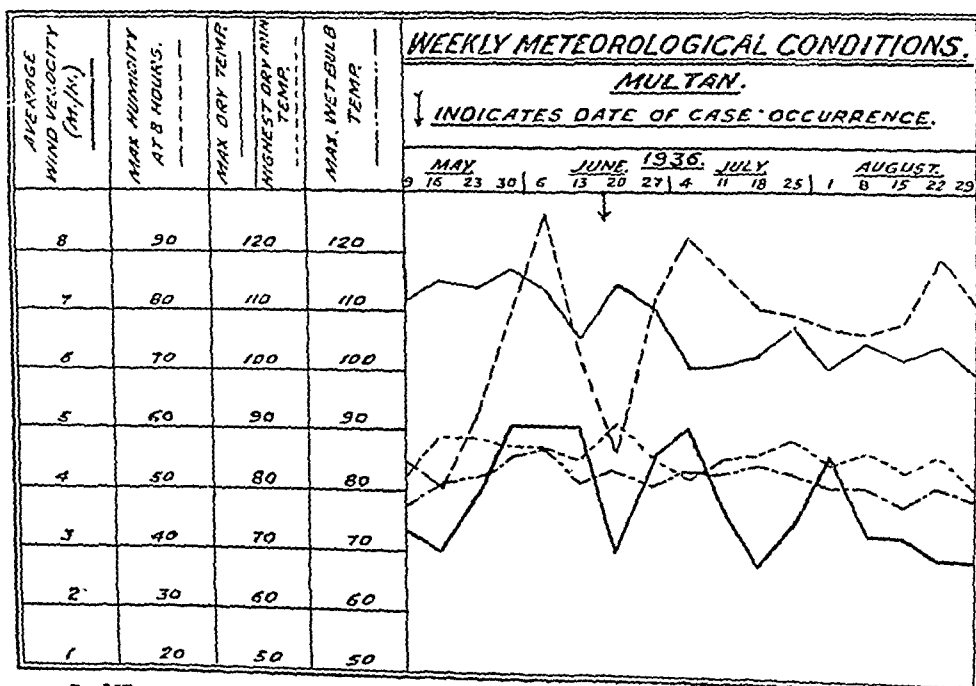
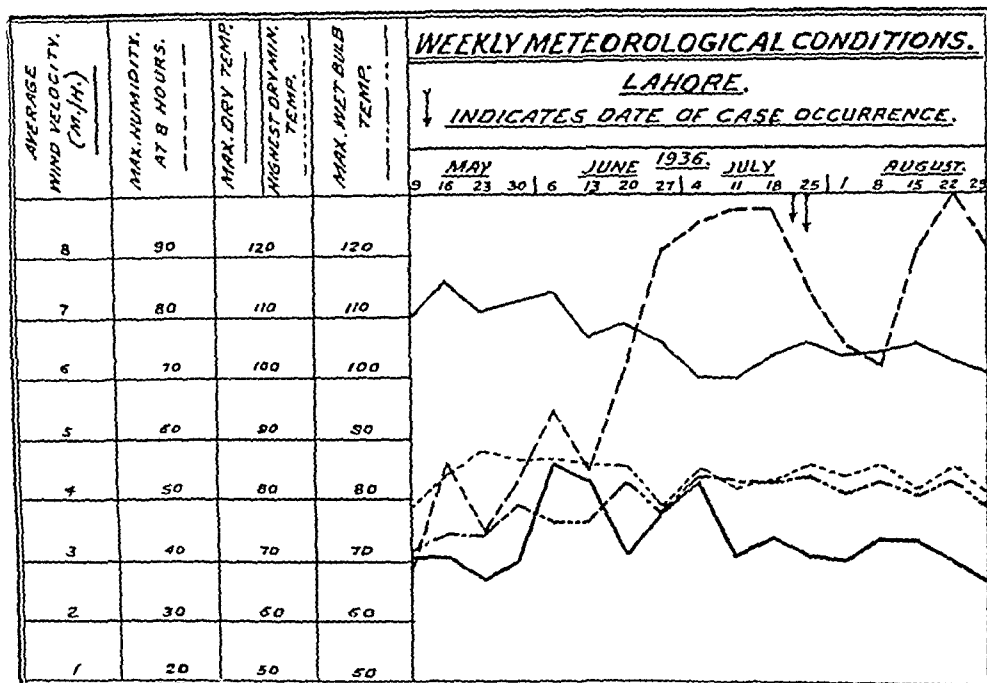
Case 10.—I. a girl aged 13 years, civilian (Indian), station Allahabad.

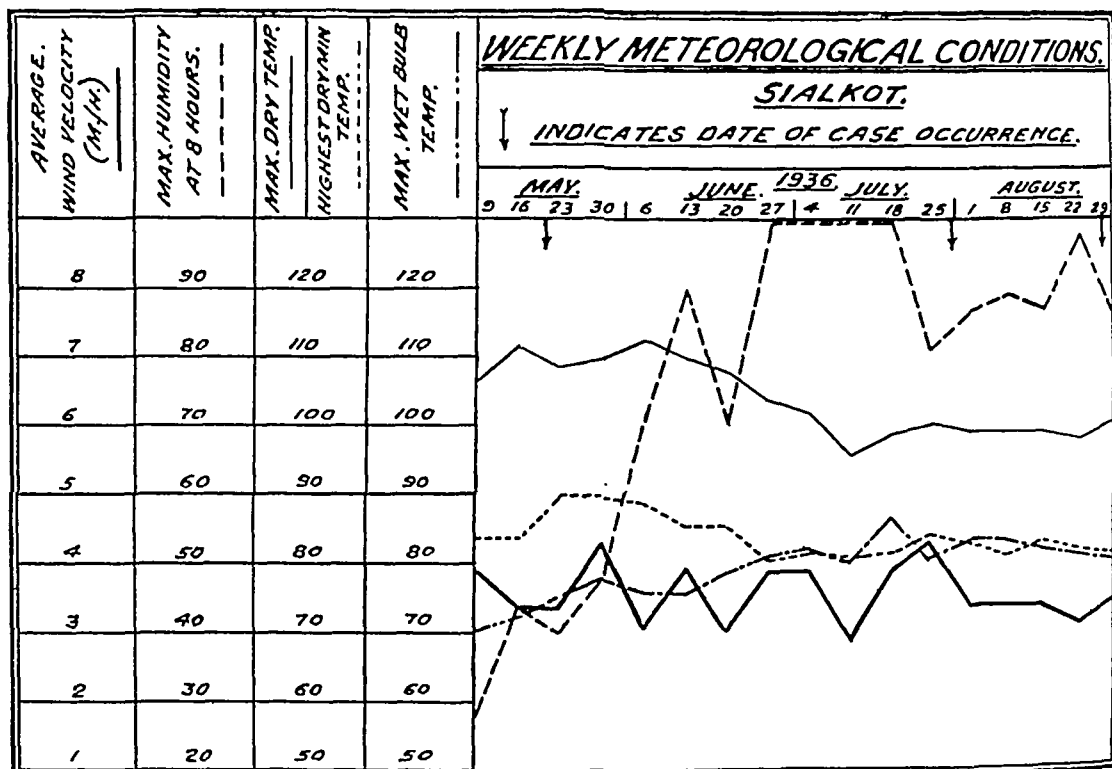
A fatal case of heat stroke, brought to hospital by the parents with a history of exposure to the sun during the day. Maximum temperature recorded 107°F . Blood smears negative for malaria parasites. Duration of pyrexia 20 hours (in hospital) ending in death.

Special signs and symptoms noted were marked cerebral irritation followed by unconsciousness, gastric tetany with carpopedal spasm and spasmodic contractions of muscles, reflexes absent, Babinski extensor, and breathing stertorous.

APPENDIX C.







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NOTICE.

Fifth Course of Post-graduate Instruction in Malariology, under the auspices of the League of Nations, Singapore.

April 1938.

THE League of Nations is arranging for a Fifth Course of Instruction in Malariology which will commence at the King Edward VII College of Medicine at Singapore in April 1938.

The object of the Course is to complete the training of medical practitioners who are engaged, or intend to be engaged, in the work of the malaria control in their own countries. The Course will thus be of interest not only to Governments and Municipal authorities, but also to all medical men practising in Eastern countries, particularly those engaged in estate work.

The Course will continue for a period of six to seven weeks and will be carried out partly at the King Edward VII College of Medicine and partly at the Tan Tock Seng Hospital, Singapore.

The practical Malaria Surveys will be arranged for outside Singapore at the end of the Course in a suitable district in Malaya where the principal vectors are found and where their habits can be studied as well as the measures adopted to control their development.

Conditions of admission.—The Course is open to medical practitioners, from whom applications for admission will be received up to the 26th February 1938.

Fee.—The fee for the whole Course or any part of it is Seventy-five Straits Dollars (\$75), payable in advance.

General.—The Syllabus for 1937 (enclosed) gives a general idea of the arrangement of the subjects and the time allotted to each. The lecturers for 1938 will not necessarily be those who assisted in 1937.

—Editor.

SYLLABUS FOR 1937*.

THEORETICAL, CLINICAL, AND LABORATORY STUDIES WITH
PRACTICAL DEMONSTRATIONS.

19th April to 29th May, 1937.

I. Chemotherapy :—

Syllabus.—Historical review. Therapeutic specificity. Chemical structure and relationship of the important malarial remedies.

A. *The Cinchona alkaloids.*—Quinine :—Structure ; pharmacology ; range of action ; dosage, methods of administration, etc. ; schemes of quinine treatment ; the relapse problem. Individual alkaloids : Quinidine ; Cinchonine and Cinchonidine. Alkaloidal mixtures : Cinchona febrifuge ; Totaquina. Quinine compounds and derivatives : Hydroquinine ; Tebetrin ; Malarcan ; Giemsa 77 ; Quiniosovarsol, etc.

B. *Miscellaneous remedies.*—Synthetic schizonticides : type Atebrin ; pharmacology ; toxicity ; dosage, etc. ; practical application in prophylaxis and treatment. Synthetic gametocides : type Plasmoquine ; pharmacology ; toxicity ; dosage, etc. ; practical application in prophylaxis and treatment.

Chemoprophylaxis : theoretical consideration ; clinical prophylaxis ; gametocyte prophylaxis ; practical applications. i. Quinine ; ii. Atebrin ; iii. Plasmoquine.

II. Clinical :—

A short course including two lectures and two ward sessions of three hours each commencing on Monday, 24th May, 1937.

Syllabus.—Demonstration of selected cases and discussion. Relapses and resistance to malaria ; provocation of attacks. Induced malaria ; delayed manifestations ; complications and sequelæ. Toxicology in relation to the use of quinine and other drugs. Determination of what constitutes a cure. Blackwater fever.

III. Control :—

A series of lectures and lecture-demonstrations will be given from April 21st until the completion of the course, occupying a minimum period of 60 hours. Field demonstrations on urban malaria control will be arranged through the courtesy of the Municipal Health Officer, Singapore. Opportunity will be given to students to familiarize themselves with field conditions. Malaria surveys will be carried out by the graduates themselves in conjunction with the lecturer on Entomology.

Syllabus of lectures.—Malaria surveys ; the measurement of malaria in the field. The history of malaria control in Singapore. The organization of anti-malarial services in urban and rural areas. The classification and application of malaria control measures. Species control and the choice of methods suitable to a particular locality. The influence of biological knowledge on the control of malaria. The limitations and uses of larvicides. The importance of drainage. Temporary (recurrent) and permanent control measures, their efficiency, cost, and financial benefit. Anti-malarial legislation and propaganda.

IV. Entomology :—

Brief syllabus.—The general morphology, life-history and importance of the Hexapoda. The Diptera Nematocera ; the family Culicidæ, its sub-families, tribes, and important genera. The genus *Anopheles*, its classification and affinities ; the species and categories below the species. The detailed morphology, life-history, habits, distribution, and oecology of *Anopheles*. Susceptibility ; influences determining the efficiency of carriers. The determination of species. The technique of laboratory and field investigations.

V. Epidemiology :—

Syllabus.—Introduction. The geographical distribution of malaria, and its social and economic importance. Definitions—endemic, epidemic, incidental, imported, and indigenous malaria.

* Subject to minor alteration in accordance with the views of the lecturers participating in the course.

Malariometry. Determination of presence of malaria; methods of estimating the amount of malaria; necessity for accurate measurements; spleen census; parasite and other indices.

Endemic malaria. The incidence and fundamental aetiology of malaria—infected, susceptible and insect hosts; factors relating to the infected human host—species and prevalence of parasites; gametocytes; infectivity; immunity; treatment; environmental factors. Factors relating to the insect host—species and prevalence of anophelines; species susceptibility and infectivity; androphilism; zoophilism; deviation of anophelines; anophelism *sine* malaria; environmental and climatological factors. Factors relating to the susceptible human host—susceptibility, natural tolerance, immunity, etc.; accessibility; quantum of infection; economic, social, and environmental factors; prophylaxis and treatment.

Climatology and malaria. Influence of temperature, humidity, wind, rainfall, and other factors. Special malaria problems—urban malaria, rural malaria, man-made malaria, malaria and irrigation, malaria and rice cultivation, etc.

Epidemic malaria. Definitions and characters—seasonal, localized, tropical aggregation of labour, regional and fulminant epidemics. Fulminant epidemics—characters, causative factors, forecasting, precautionary and remedial measures.

The spleen in malaria. Enlarged spleen—age; degree of enlargement; relation to degree of parasitaemia, frequencies of enlarged spleens; theories, significance in epidemiology.

Malaria surveys. Definition; when and why undertaken; what surveys do; scope of surveys and staff required; sources of information; methods of investigation; general conduct of surveys. Laboratory and field studies; vital statistics; analysis of data collected; interpretation of results; recommendations.

VI. Malarial Haematology and Protozoology :—

Syllabus.—The normal red cells of human blood and their variants in health and disease. Comparative red cell morphology of mammals, birds, and reptiles. The preparation and staining of blood films and tissue smears for malarial parasites. The Romanowsky process. The preparation of Leishman and Giemsa stains. The counting of blood cells and parasites. The Sinton-Dreyer fowl-corpuscle technique. The estimation of haemoglobin.

Classification of the Protozoa. The developmental cycle of the malarial parasite.

***Plasmodium falciparum* :** Development. Morphology in fresh blood, in stained thick and thin films and in tissue smears. Peculiarities of localization in the tissues. Biological and morphological variation. Species and sub-species not universally recognized.

***Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* :** Development. Morphology in fresh blood and in stained thick and thin films.

The parasites of simian and avian malaria.

Prognosis in relation to parasitological findings. The culture of malarial

VII. Pathology and Immunity :—

Syllabus.—Pathogenicity of different malaria parasites and the defence mechanism of the vertebrate host. Nature of the malaria paroxysm; virulence of malaria parasites; susceptibility of host; natural and acquired immunity (resistance, tolerance, premunition, etc.)—definitions and general considerations.

Pathological changes in acute pernicious malaria. Changes in various organs and tissues; localization of parasites; malaria 'toxins'; correlation between pathological changes and clinical syndromes.

Pathological changes in benign and chronic malarial infections. Changes in various organs and tissues at different stages of the malarial infection (early crisis, post-crisis, chronic, latent); pathological changes in superinfections.

Infection and superinfection with different species and strains of malaria parasites. 'Strains' of malaria parasites—human, simian, avian; specificity of strains under experimental conditions; importance of strains in relation to malaria therapy and epidemiology.

The defence mechanism in malaria. Cellular basis for immunity; antibodies; interference with immunity by experimental procedures; duration of immunity; relapse; immunity in relation to the treatment of malaria.

Laboratory studies. Study of selected microscopical material illustrating the tissue changes in malaria.

VIII. Special Lectures : —

1. Lecture-demonstration. The Physical Properties of Oils used as Larvicides.
2. Malaria in Indo-China.
3. The Work of the Malaria Commission of the League of Nations.
4. Immunity in Malaria.
5. Malaria in India.

NOTICE.

The following has been received for publication :—

—Editor.

UNIVERSITY OF LONDON.

UNIVERSITY READERSHIP IN MEDICAL PARASITOLOGY TENABLE AT THE LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE.

THE Senate invite applications for the University Readership in Medical Parasitology tenable at the London School of Hygiene and Tropical Medicine.

It is desirable that candidates should be competent in medical protozoology and should have had experience overseas.

Applications, accompanied by the names and addresses of not more than three persons to whom reference may be made, should reach the Academic Registrar, University of London, W.C.1, not later than the first post on Friday, 18th February, 1938. If testimonials are submitted, copies of not more than three should be sent. Twelve copies of all documents must be furnished. Envelopes should be marked 'Readership in Medical Parasitology'.

Candidates naming as references persons resident abroad should ask such persons to write direct to the Academic Registrar without further intimation.

Candidates (other than those resident abroad) who are summoned for interview will probably be asked to attend at the University on an afternoon in the week beginning 28th February, or in the following week.

Under the University Regulations Boards of Advisors are not limited in their choice to persons who have applied for the post (*see* University Calendar for 1937-38, p. 267).

15th December, 1937.

S. J. WORSLEY,
Academic Registrar.

Title :—

1. The full title of the holder of the Readership will be 'Reader in Medical Parasitology in the University of London'. The Readership is attached to the London School of Hygiene and Tropical Medicine.

Tenure :—

2. The appointment will date from 1st April, 1938, or as soon thereafter as may be found practicable and will be subject to the Statutes and Regulations of the University and to the rules of the London School of Hygiene and Tropical Medicine in regard to such appointments.

3. The Senate will decide after report from the relevant Board of Advisors whether the appointment shall be made in the first instance for an initial limited period, and if so what period, or whether it shall be made without time-limit up to the age fixed for retirement. If the appointment is renewed after an initial period it will be tenable up to the age fixed for retirement as prescribed in Clause 4 below.

4. The Reader will normally be required to retire on 30th September in the session in which he attains the age of 60. The Senate may, however, acting on the advice of the Governing Body of the School, invite him to continue to hold office for a further period up to the age of 65.

5. The Reader shall not resign his Readership, unless for special reasons approved by the Senate, except as from the close of the academic year (30th September) and after giving, on or before 30th April preceding, notice of his intention to resign.

6. The appointment will be a whole-time appointment and the Reader shall not hold any other public appointment or engage in any other professional work without the approval of the Board of Management of the School.

7. The Reader will be entitled to two months' holiday in the year.

Salary :—

8. The salary of the Reader will be £900 a year provided by the London School of Hygiene and Tropical Medicine.

Superannuation :—

9. The Reader will be required to contribute 5 per cent of his salary to the Federated Superannuation System for Universities, the Board of Management contributing from the School funds an amount equal to 10 per cent of his total remuneration.

Duties :—

10. The Reader will be a member of the Department of Parasitology, at the London School of Hygiene and Tropical Medicine, of which the William Julien Courtauld Professor of Helminthology (Prof. R. T. Leiper, F.R.S.) is the Director.

11. It will be his duty to assist the Professor in the teaching, organization and general conduct of the Department; and to do all in his power to advance the subject of Parasitology, especially in relation to Medical Protozoology, by teaching and research. He may be required by the Board of Management to proceed overseas from time to time for periods of study and research. Special travelling allowances will be granted in respect of these periods.

12. The Reader will be an Appointed Teacher of the University and a member of the University Faculties of Science and Medicine and of the appropriate Board, or Boards, of Studies. He will also be a member of the School Council of the London School of Hygiene and Tropical Medicine. His attendance at meetings of Boards and Faculties of which he is a member will be considered an important part of his work.

ATTEMPTS TO CULTIVATE *M. LEPRÆ MURIS*.

BY

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AND

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Fund Association.*

(From the School of Tropical Medicine, Calcutta.)

[Received for publication, December 31, 1937.]

IN this Laboratory we have been attempting to confirm some recent claims of success in the cultivation of the organisms of human and rat leprosy. We have tried to grow the organisms in tissue cultures, in minced chick tissue medium, on split-protein medium, on the different media in the gaseous environment recommended by Soule and McKinley, and from the blood of leprosy patients as advocated by Lowenstein. In all during the last year about two thousand media tubes and flasks and eight hundred pieces of tissues have been seeded. Every inoculated media tube and piece of tissue has been examined by means of smears taken after it has been incubated for a certain period.

The material used for inoculation.—Most of our work has been done with rat-leprosy material because it is easy to obtain and also because the genuineness of any culture obtained can be proved by animal inoculation. Emulsions made from spleens, omenta, and lymph glands of rats in the advanced stages of the disease experimentally produced were used for seeding the media. In some of the experiments these emulsions were treated with 5 per cent sulphuric acid and then neutralized, while in others no such treatment was given.

Our attempts to cultivate the organisms of human leprosy as advocated by Lowenstein have largely been confined to the use of blood of patients with leprosy; in one experiment the media were seeded with emulsion from subcutaneous nodules from a patient.

The media used were :—

Egg media.—The media of Hohn, Petraghini, Petroff, Twort and Wherry, and glycerine-egg medium, glycerine-egg media with addition of emulsion of rat-leprosy bacilli killed by heat.

Glycerine media.—Glycerine agar, hormone-glycerine agar, glucose-glycerine agar, and potato-glycerine agar.

Potato media.—Glycerol potato, glycerol-broth potato, and Shiga's medium.

Split-protein medium of Duval and Holt, containing tryptophane, cystine, tyrosin, leucine, placental 'autolysate', and banana infusion in glycerine agar.

Fish media.—Fish-broth medium of Lowenstein. Fish-egg agar.

Media made from rat spleen.—Rat-spleen agar. Pieces of rat spleen in glucose broth, glycerine broth, and potato broth.

Tissue cultures.—Pieces of rat spleen, liver and skin, rat-bone marrow, and chick leucocytes.

As the work reported in the present paper deals mostly with the attempts at verifying the claims of various workers to have cultivated leprosy bacillus by different methods, we propose to give a short review of the literature of each method and then to give our own results by that particular method.

SPECIAL GASEOUS ENVIRONMENT.

The attempts to cultivate the leprosy bacillus under special gaseous environment are based on the work of Wherry and Ervin (1918) who showed that carbon dioxide was essential for the growth of *M. tuberculosis*, and on the extensive studies of Novy, Roehm and Soule on microbial respiration with special reference to the respiration of the tubercle bacillus. Wherry (1930) used semi-solid media prepared by the addition of glycerinated ovomucoid yolk solution to nutrient agar. The tubes were seeded with a loopful of blood expressed from a leprosy nodule containing numerous lepra bacilli. These tubes were then incubated under various gaseous conditions. Proliferation of the bacilli was noted in three cases after a few weeks. The best growth was obtained in cultures which were kept first at low oxygen tension (little O_2 but CO_2 present) for a month, after which oxygen was increased.

Soule and McKinley (1932) claim to have cultivated *M. lepræ* under special gaseous tension. Tubes of glycerol potato, glycerol-broth potato, Petroff's and Dorsett's egg media, glycerol agar, hormone-glycerol agar, etc., were seeded with emulsions made from aseptically removed leprosy nodules. The tubes were placed in Novy jars in atmospheres containing various mixtures of nitrogen, oxygen, and carbon dioxide and some were kept under ordinary atmospheric conditions. All these tubes were inoculated at 37.5°C. After six weeks of incubation it was noted that no growth took place in the tubes incubated under ordinary atmospheric conditions and in oxygen-free jars. Growth was detected in some of the tubes incubated under atmospheres containing both O_2 and CO_2 and it was noted that the most favourable gaseous environment was 40 per cent O_2 and 10 per cent CO_2 . Of the media used, glycerol potato, Petroff's medium, and hormone-glycerol agar gave the best results. In 46 tubes there were present small colonies with 'a distinct mucoid appearance and a loose filamentous border'. Stained smears from these colonies showed well staining acid-fast rods. These organisms were carried through 16 generations but there was a gradual loss of the growth at each subculture; there were 46 positive cultures in the first generation, while in the sixteenth generation only two positive tubes were left.

Soule (1934) got positive results in 12 out of 20 specimens of human leprosy material, the culture tubes being incubated in a gaseous environment containing 40 per cent O_2 and 10 per cent CO_2 . He describes tiny, flat, transparent, pin-head colonies which never became large. The growth was viable through three generations. To rule out the possibility of these appearances being produced by the mere mechanical transference of bacteria, control tubes were inoculated with material which had been autoclaved, but similar colonies were not observed.

The work of Wherry, of Soule and McKinley, and of Soule has not been confirmed by other workers who employed similar methods of cultivation. Oliver, Leon and Roda (1931), using Wherry's method, did not note any macroscopic or microscopic evidence of proliferation of the bacilli. Duval and Holt (1934a) inoculated a variety of solid and liquid media with material from subcutaneous nodules and leprosy abscesses and incubated by the method of Soule and McKinley. No proliferation of *M. leprae* was noted after several weeks' incubation. Duval and Holt think that the positive cultures and microcolonies of Soule and McKinley may possibly be explained by the fact that *M. leprae* may continue to proliferate in leprosy tissue that has been removed from the human host. The statement that the growth became less and less in successive subcultures and subsequently ceased is quoted in support of this view. They consider this lessening of growth and its ultimate cessation to be due to the transferred culture inoculum containing a decreasing amount of the original tissue products of protein digestion.

Present work.—We inoculated 1,205 tubes of the different media in 27 experiments. Six hundred and eleven of these tubes were incubated under 40 per cent O₂ and 10 per cent CO₂ and the remaining 594 tubes under ordinary atmospheric conditions. The special gaseous tension was produced in vacuum desiccators which were re-gassed about every month.

All the tubes were examined about once a month for three or four months. At the first and second examinations smears were taken from some of the tubes. At the final examination smears were taken from all the tubes and from some of the tubes two or more smears were taken from different sites.

Of the 1,205 tubes 260 tubes showed contamination with staphylococci, non-acid-fast rods, spore formers, etc. Two hundred and thirty-nine tubes showed no organisms at all, 642 tubes showed few or a moderate number of acid-fast bacilli, and 64 tubes showed a large number of acid-fast bacilli. Of these 64 tubes 38 had been incubated in the special gaseous environment and 26 had been incubated under ordinary atmospheric condition. In these 64 tubes there was some macroscopic and considerable microscopic evidence of multiplication, the bacilli being found in very large numbers, sometimes in masses. However, subcultures from these tubes showed a steadily decreasing number of acid-fast bacilli up to the third generation when subcultures were stopped.

Conclusion.—While in many of the primary culture tubes we observed appearances suggesting multiplication, we do not consider that these findings give *conclusive* evidence of multiplication of the bacilli, or of the value of the special gaseous environment in obtaining multiplication but we think that the matter should be further studied.

TISSUE CULTURE.

Zinsser and Carey (1912) were the first to employ tissue culture for the cultivation of leprosy bacillus. They used small pieces of spleen from rats not more than one week old and cultivated them in rat plasma. These tissues were infected with rat-leprosy bacilli from rats suffering from rat leprosy. The bacilli increased in small numbers in the cells but no growth was obtained outside the cell and attempts to transfer the organisms to laboratory media were not successful.

Friedheim (1929) did not observe any multiplication of rat-leprosy bacillus in cultures of rat spleen planted in rat plasma and rat-embryo extract.

Timofejewsky (1929) claims to have obtained multiplication of the leprosy bacilli in cultures of fragments of leprosy nodules planted in human and rabbit plasma and human-embryo extract. The cultures died off after a few weeks but the bacilli increased for a time even after the tissue died. When the bacilli from such old cultures were transferred to cultures of healthy human tissue and of leucocytes further multiplication of these bacilli was obtained.

Denney and Eddy (1933) subjected the acid-fast bacilli in 'lepra juice' obtained from an incision in a nodule and from pus from leprosy abscesses, to the influence of living leucocytes obtained from

artificially-produced pleural effusions in rabbits. The bacillary suspension and the leucocytes, after being thoroughly mixed, were drawn up in a narrow pipette which was sealed and incubated at 37°C. At appropriate intervals the contents were examined by blowing out a droplet on to a microscopic slide by breaking open the thin end of the pipette, the pipette being sealed again. Denney and Eddy did not obtain any evidence of proliferation of either free acid-fast rods or of globi contained in 'lepra juice'. In the pus obtained from leprosy abscesses they observed proliferation of the acid-fast bacilli as shown by an increase in the number and size of globi.

Salle (1934) cultivated a mixture of acid-fast and non-acid-fast organisms from four 'broken down' nodules obtained from two patients of leprosy and in one instance from rat-leprosy material on chick-embryo tissue planted in heparinized guinea-pig plasma and chick-embryo extract. He has described the growth of these organisms in alternating acid-fast and non-acid-fast phases, the organisms being acid-fast in actively-growing tissues and non-acid fast in dead and autolyzed tissues and in laboratory media.

Soule (*loc. cit.*) got slow but definite growth of leprosy bacilli in tissue cultures.

Present work.—We have attempted to grow *M. lepræ muris* in cultures of pieces of spleen, liver, and skin of rat embryos, in cultures of rat-bone marrow and of chick leucocytes. Rat spleen, liver, and skin were planted in a mixture of chick plasma, chick-embryo extract and rat serum on a coverslip which was inverted on a depression slide and sealed.

Chick leucocytes are obtained from the blood drawn from the vein of a cock. The blood is taken with an ice-cold syringe into an ice-cold paraffined centrifuge tube, centrifuged in ice and the plasma is pipetted off leaving the red cells with the leucocytes layer on top. After pipetting off the plasma the tube (containing the red cells and the layer of white cells) is made to stand in an absolutely upright position. Two or three drops of chick-embryo extract are added gently to the top of the layer of leucocytes. The tube is allowed to stand for about half an hour at room temperature. At the end of this period the white cells form a coagulum. This coagulum is separated from the underlying red cells with the help of a sterile cataract knife. The top of the tube is then flamed and the tube is tilted over a watch-glass containing a small amount of Tyrode's solution so that the leucocyte film slips into the watch-glass. The film is gently moved to and fro in the solution to wash off the adherent red cells. It is then placed on a sterile slide and cut into small fragments. These fragments are dipped in an emulsion containing rat-leprosy bacilli for half an hour. These are then washed in Tyrode's solution and planted in chick plasma and chick-embryo extract on coverslips.

The bone-marrow cultures are made as follows:—

The femur bones of an adult rat are removed aseptically and cleaned of all the adhering fragments of muscle, etc. Small amounts of Tyrode's solution are taken in about half a dozen sterile centrifuge tubes fitted with rubber corks. The bone is broken open longitudinally with scissors and the marrow is scraped out with an old cataract knife and put into the small centrifuge tubes. About six tubes can be made from the marrow of the two femurs of a rat. To the tubes is then added a mixture of rat serum and chick-tissue extract. A drop or two of an emulsion rich in rat-leprosy bacilli is then added to each of the tubes. Smears are made from each of the tubes after thoroughly shaking and stirring the mixture with a platinum loop. The smears are examined and the number of acid-fast bacilli found per 50 or 100 fields is noted for comparison with later observations. For the purpose of a subculture the tubes are centrifugalized, the fluid is pipetted off without disturbing the deposit and is replaced by a fresh mixture of rat serum and chick-tissue extract. The volume is then made up with Tyrode's solution.

Results.—About 600 pieces of spleen, liver, and skin from rat embryos have been cultivated and infected with rat-leprosy bacilli by being immersed in an emulsion containing the organisms for about half an hour before being planted. In a very small number of these cultures there was some evidence but no definite proof of multiplication.

About 225 pieces of chick leucocytes have been cultured and infected with *M. lepræ muris*. In 12 of these cultures (in two experiments) there was a strong suggestion of multiplication after the tissue had been kept alive for from seven to nine days. In these particular experiments the pieces of leucocytes smeared on the first day showed from 22 to 80 bacilli per piece. All the 12 pieces smeared after the seventh to ninth day showed appearances suggesting a great increase in the number of bacilli, the bacilli being found in large masses, some intra-cellular and others extra-cellular. Subcultures were made from these specimens into fresh pieces of leucocytes. In these subcultures no definite evidence of multiplication has been obtained. This work is being continued.

About 75 tubes of bone marrow have been infected. In about a quarter of the tubes some evidence of multiplication has been obtained, the bacilli showing an apparent increase in number, but no such evidence has yet been obtained in subcultures into fresh bone marrow. The experiments are being continued.

Conclusions.—In ordinary tissue cultures we have been unable to obtain definite evidence of multiplication of bacilli. In cultures of chick leucocytes and of rat-bone marrow, we have, however, observed some evidence that the bacilli multiply. No evidence of multiplication in subcultures has been obtained but the work is being continued.

MINCED CHICK-EMBRYO MEDIUM.

The cultivation of the bacillus of leprosy in minced chick embryo was mentioned for the first time in his Annual Report for the year 1932, by the Surgeon General of the Public Health Service of the United States. No macroscopic colonies were obtained but in three out of seven attempts with human material, and in one out of three attempts with rat-leprosy material there was an apparent proliferation of acid-fast bacilli in five to seven days. The multiplication of the acid-fast bacilli was accompanied by a definite growth of a diphtheroid bacillus. The organisms were carried through several subcultures and the acid-fast bacilli in the last subculture appeared to be as numerous as in the original cultures.

McKinley and Verder (1933) described the cultivation of *M. lepræ* in minced chick-and-human-embryo tissue. There was evidence of growth and multiplication in five days and marked growth was obtained in 10 to 15 days. Growth was obtained under atmospheric conditions but the cultures grew much better under the gaseous environment of Soule and McKinley. The cultures after being grown in this medium for several generations were transplanted to solid media which were inoculated and incubated in the same gaseous environment. On the solid media definite micro-colonies of acid-fast organisms were observed. Soule (*loc. cit.*) confirmed the work of McKinley and Verder in 22 instances. Out of 26 attempts to cultivate *M. lepræ* in minced chick-embryo medium he obtained 'unquestioned evidence of proliferation'. To rule out the possibility of these appearances being produced by the mere mechanical transfer of bacteria he used control flasks inoculated with autoclaved suspension of the original material. In these control flasks it was possible to find acid-fast bacilli but their number was negligible. The positive cultures were carried through six generations in the minced chick-tissue medium. Attempts to transfer the growth to tubes of Petragnum's and Lowenstein's media did not succeed.

Salle (*loc. cit.*) used minced chick-embryo medium to propagate his cultures of *M. lepræ* and *M. lepræ muris* which had been carried for a number of generations in tissue cultures.

Holt (1934), however, failed to produce growth of *M. lepræ* in chick-embryo tissue. Holt used both liquid and solid media containing the emulsified chick tissue and incubated his cultures both

under ordinary atmospheric conditions and in Novy jars containing 10 per cent CO₂ and 40 per cent O₂. He did not note any evidence of the proliferation of *M. lepræ* and concluded that 'chick-embryo tissue emulsified in Tyrode's solution is *per se* valueless as a nutritive in the *in vitro* cultivation of *B. lepræ*'. Walker and Sweeney (1935) did not obtain multiplication of acid-fast forms of *M. lepræ muris* in minced embryo tissue suspended in Tyrode's solution, but they obtained acid-sensitive forms which on subculture on suitable media became acid-fast.

Present work.—In our experiments we used chick embryos seven to eleven days old to prepare the minced chick-tissue medium. The minced tissue was put in small tubes and diluted with about three times its volume of Tyrode's or Pannet's solution. These medium tubes were seeded with a drop of emulsion rich in rat-leprosy bacilli. A smear was taken from each tube immediately after seeding; this was stained and examined and the number of acid-fast bacilli seen per 50 or 100 fields was recorded. The inoculated tubes were incubated for a week, and then smears were again taken after thoroughly mixing the fluid. These smears were stained and examined for acid-fast and non-acid-fast organisms. The number of acid-fast bacilli per 50 or 100 fields was recorded and compared with the number recorded before incubating the tube. Seventy-five tubes in 10 experiments were thus inoculated.

The results of examination of those 75 tubes were as follows:—

No organisms	15
Acid-fast organisms only	21
„ and non-acid-fast organisms	27
Non-acid-fast organisms	12
				—
			TOTAL	75
				—

In a separate paper we have discussed the significance of these non-acid-fast forms. We are of the opinion that these non-acid-fast organisms are contaminating organisms and not the non-acid-fast forms of the leprosy bacillus. In most of the 21 tubes showing only acid-fast bacilli these organisms were very scanty and there was no suggestion of multiplication, but in a few of these tubes there was an apparent increase in the number of bacilli. Fifty tubes were used for sub-cultivating from some of these tubes. Some bacilli could be carried from one tube to another but no definite evidence of multiplication was observed.

CULTIVATION OF LEPROSY BACILLUS FROM BLOOD.

Lowenstein (1935) obtained a rich growth of acid-fast bacilli from dehaemoglobinized blood in glycerinated fish broth after four months' incubation. From this medium the growth was transferred to glycerine agar to which fish broth had been added. On this solid medium macroscopic growth was obtained in four months; there developed colonies of two descriptions, one whitish and the other of a light yellow tint. On further cultivation the whitish colonies yielded an organism which had all the characteristics of true mammalian tubercle bacillus and were identified as such. However, the small yellowish colonies at first consisted of non-acid-fast bacilli with an occasional acid-fast rod, but later there appeared long thin bacilli staining bright red with Ziehl-Neelsen's method. In the following generation the organism produced a definite moist growth on fish-broth-egg medium and glycerine-egg medium after eight weeks and after another eight weeks the growth contained large masses of acid-fast bacilli mixed with non-acid-fast bacilli identical in size and shape with leprosy bacilli.

Ota and Sato (1934) by using Lowenstein's method of blood culture obtained 12 strains of the leprosy bacilli from 83 cases. They used Petragnini's, Petroff's, Lowenstein's and glycerol-potato media, the best growth being obtained on the Petragnini's medium. Their cultures were of two types, white or creamy white (*typhus albus*) and ochre yellow or orange (*typhus auranticus*). These two types were not definite as there was an intermediate type also and the colour was changeable

from white to orange, and vice versa. A culture of the yellow type was obtained from a nodule taken from a patient from whose blood a growth of the same type was obtained. In another case two identical strains were obtained from the blood of a patient on two different occasions. Ota and Sato consider these two findings of importance in affording evidence for the identification of the bacillus. Lleras (1935) inoculated Petraghini's medium with acid-treated dehaemoglobinized blood from leprosy patients. He obtained large, yellowish humid colonies composed of acid-fast granular rods in globi after ten days' incubation. Injection of this culture to guinea-pigs did not produce tuberculosis. Fifteen successful subcultures have been made.

De Souza Araujo (1933) failed to confirm the positive blood culture results of Ota and Sato. He obtained 26 samples of blood from 17 lepers with active disease. Each of these specimens was used for seeding Petraghini's, Petroff's, Lowenstein's, glycerine-potato and glycerine-broth-agar media. In none of the media was any growth obtained during six months' incubation.

Present work.—We obtained specimens of blood from 15 cases of leprosy, nine were nerve cases, six cutaneous cases. (Leprosy bacilli were found in smears of the dehaemoglobinized blood in one of the nerve cases in the phase of reaction and three of the cutaneous cases). Ten cubic centimetres of blood were removed from a vein at the bend of the elbow; this was received into two sterile centrifuged tubes containing about 1.5 c.c. of 3 per cent sodium citrate solution. Both the tubes were centrifugalized and the deposit, after removing the supernatant fluid, was dehaemoglobinized by the addition of 3 per cent acetic acid. The tubes were again centrifugalized, the deposit from one of the tubes being used for incubating the media without further treatment while the deposit in the other tube was treated with 5 per cent sulphuric acid before being so used. In addition to fish broth as suggested by Lowenstein, flasks of papain broth and tubes of split-protein medium, Petroff's, Hohn's, Petraghini's media and hormone-glycerine agar, etc., were seeded with acid-treated and non-acid-treated dehaemoglobinized blood. In all 30 flasks and 430 tubes were used. The tubes and flasks were examined from four to six weeks after being seeded; they were re-examined after another 12 weeks. No evidence whatever was obtained of the growth of the leprosy bacillus from the blood of the patients in these media. Only in two of the tubes examined were scanty acid-fast bacilli seen.

SPLIT-PROTEIN MEDIUM.

Since 1910, Duval has been expressing the view that *M. lepræ* seemed unable to utilize whole proteins in culture media and that to get growth of this organism it is essential to incorporate split-protein products in the culture medium. He has advocated two methods of accomplishing this end. In the 'direct' method tryptophane or a mixture of albumin and trypsin is incorporated in the culture medium. In the 'indirect' method bacteria capable of digesting the albumin constituents of the culture medium are introduced into the medium.

Duval and Holt (1934b) have described an 'improved method for *in vitro* cultivation of *B. lepræ*'. Aseptically removed subcutaneous nodules rich in acid-fast bacilli are cut into small pieces, placed in a 1 per cent trypsin solution, and incubated at 37°C. from 36 to 48 hours. During this period the protein in the nodule is digested and its split products are said to be utilized by *M. lepræ* for its growth. After this preliminary incubation the softened nodules are put on the surface of amino-acid-agar tubes and spread with a platinum loop. These culture tubes are then incubated in the usual manner.

Present work.—Small pieces of spleens from rats suffering from experimentally-produced leprosy were digested with 1 per cent trypsin solution for from 96 to 120 hours. The softened tissue was then inoculated on the different media including the split-protein medium of Duval and Holt. In all 70 tubes were inoculated. In one experiment smears from all the 26 tubes of the split-protein medium showed enormous numbers of bacilli suggesting multiplication. Subcultures from these tubes were made into a large number of tubes. Smears from the subculture tubes

were taken before they were put in the incubator. The number of acid-fast bacilli found per 50 fields in these smears was compared with a similar examination made after the tubes had been in the incubator for eight weeks. No appreciable increase in the number of bacilli was found.

SUMMARY.

1. Details are given of attempts to confirm the findings of workers who have in recent years claimed to have cultured the organisms of human and rat leprosy.
2. No conclusive evidence has been obtained of the value of a special gaseous environment as recommended by Soule and McKinley, in promoting multiplication of the bacillus on artificial medium.
3. In ordinary tissue cultures no conclusive evidence of multiplication of bacilli has been obtained.
4. In cultures of chick leucocytes and of rat-bone marrow, some evidence of multiplication has been obtained, but this has not been verified by subculture.
5. No conclusive evidence of multiplication of bacilli in minced chick-embryo medium has been obtained.
6. Attempts to culture the organism of leprosy from the blood by the method of Lowenstein have given negative results.
7. No conclusive evidence of multiplication of bacilli in cultures on split-protein medium as recommended by Duval has been obtained. In primary cultures there was some evidence of multiplication, but this has not been verified by subcultures.

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A SEROLOGICAL STUDY OF THE ACTINOMYCES*.

BY

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THE microbiological studies have so far failed to assign a definite place to actinomyces in systematic bacteriology, whether they should be classified with the schizomycetes or hyphomycetes is still an open question. If the presence of an antigen common to actinomyces and other well-known bacteria could be established, then it would lend strong support to the classification of actinomyces as schizomycetes. The complement-fixation test can be usefully employed in the elucidation of this problem. Claypole (1913) concluded from her work that although there was an antigen common to the actinomyces (acid-fast and non-acid-fast) and acid-fast bacilli, yet the degree of complement-fixation varied; homologous antigens could be differentiated from the heterologous ones by this means. Boquet, Negre and Urbain as quoted by Calmette (1936) found an antigen common to tubercle and diphtheria bacilli. Boquet and Negre (1923) found in addition that methyl alcohol extracts of *Bacillus subtilis* and Rivolta's organism could bind the complement in presence of anti-tuberculous sera, but the degree of this fixation was not high. Urbain (1922) found that streptococci reacted with anti-tuberculous serum by fixing 15 units of antibody, whilst the homologous antigen fixed 1,200 units. Urbain and Fried (1921) observed only very occasionally an interaction between the human anti-tuberculous serum and *Bacillus subtilis*, but the degree of this activity was unfortunately not indicated. The last observation could not be confirmed by Dienes and Balas (1924). Furth and Aronson (1927) could differentiate avian tubercle bacilli from the mammalian by serological tests. Rice and Reed (1932) found that only smooth strains had a specific antigen. Schaefer (1935) stated that the R variant of a strain of avian bacilli contained an antigen which was common to all acid-fast bacilli, whereas the S variant contained both specific and group antigens. It appeared from the work of the above-mentioned authors that the group antigen was found not only amongst acid-fast bacilli but amongst certain non-acid-fast strains also. Bretey (1933) described an antigen common to

* This paper summarizes part of the work done at the Pasteur Institute, Paris, in recognition of which the Academie des Sciences and the Academie de Medicine of Paris have awarded the author the 'Mention Honorable Montyon' and the 'Encouragement Audiffred', respectively.—Ed.

actinomyces and acid-fast bacilli, but found the actinomyces antigens to be very poor in their action both *in vivo* and *in vitro*. It is considered that the weak sera obtained by Bretey (*loc. cit.*) might have been due to the injection of small doses of antigen administered at rather infrequent intervals.

In view of the unsatisfactory state of knowledge of the antigenic relationship between the actinomyces and acid-fast bacilli, the author undertook a critical study of the problem at the Pasteur Institute, Paris.

All the strains of the actinomyces at disposal were unemulsifiable, hence the use of the aqueous emulsion was out of question. Methylic extracts prepared according to the technique devised by Boquet and Negre were utilized.

The sera were prepared by giving 4 to 8 intravenous injections of 20 mg. to 100 mg. of living actinomyces to rabbits at 3- to 7-day intervals. The animals were bled 7 to 15 days after the last injection and the sera obtained from the same group were pooled. The dose and interval of injection were varied according to the resistance of the animal. In case of loss of weight, the interval was prolonged and the dose was reduced. Rabbits looking ill after the last inoculation had to be sacrificed earlier than others.

The antibody was estimated by the technique of Calmette and Massol, 1 c.c. of 1/20 dilution of the methylic antigen being employed.

Use was also made of other antisera besides the anti-actinomycotic, i.e., tubercle, para-tubercle, and diphtheritic serum. Some of these antigens and antisera were prepared by W. Schaefer, to whom we are much indebted. He inoculated intravenously into rabbits 5 to 10 times 5 mg. to 10 mg. of heat-killed bacilli, the animals being sacrificed 6 days after the last injection.

Bretey (*loc. cit.*) gave 5 intravenous injections at 5-day intervals into rabbits of 20 mg. of actinomyces per dose and was able to demonstrate only 50 to 100 units of antibody. (If 0.1 c.c. of a 1/10 dilution of antiserum fixed 1 M. H. D. of complement in presence of a fixed amount of antigen, then 1 c.c. of undiluted serum fixed 100 M. H. D. of complement, this being the calculation adopted by Calmette and Massol.) The author obtained sera containing very little of the antibody, but 400 units were obtained from the antiserum of one rabbit, which received 9 injections of 20 mg. to 40 mg. of actinomyces. The streptothricine (an extract of actinomyces prepared exactly in the same manner as tuberculin produced by the Tuberculin Section of the Pasteur Institute) was not found to be suitable as an antigen. The anti-actinomycotic serum revealed 400 antibody units when methylic extract was used, but the streptothricine diluted 1/10 or 1/20 fixed only 100 units. In another experiment the methylic extract was found to give thrice as good results as streptothricine.

The experiments were repeated two or three times following the same technique. The number of units of antibody fixed varied slightly from one experiment to another as the titration of the complement could not be done absolutely correctly in each experiment.

The following antisera were used in this study :—

Anti-tuberculous (avian and mammalian, both rough and smooth).

Anti-paratuberculous (both rough and smooth).

A particular type of anti-tuberculous serum (smooth).

Anti-actinomycotic.

Anti-diphtheritic.

The results demonstrated the presence of an antigen common to all these bacilli. The actinomycotic antigens were found to be as active against the homologous sera as against the heterologous ones except for the following strains: Paratubercle-crottin S, Rabinowitsch-butter S, and avian A smooth. These three strains definitely showed the presence of specific antigens. The corresponding antigens fixed at least 240 to 270 units, whilst the heterologous antigens fixed only 0 to 90 units.

Griffith (1925) was able to differentiate between the mammalian and avian strains by agglutination tests, but he utilized only the smooth strains of avian bacilli. These three strains containing specific antigens were also smooth. But the strain Serpent R (belonging to acid-fast group of bacilli of cold-blooded animals) and M (a special type isolated and described by Griffith, 1933) were rough and contained nevertheless specific antigens. R. Laporte very kindly placed at our disposal R and S strains of bovine tubercle bacilli and the corresponding antisera. We were unable to differentiate serologically between the rough and smooth strains, the only point of interest being that the antiserum S contained far greater amounts of antibody than antiserum R.

With regard to the diphtheria group, the antiserum prepared with washed bacillary bodies gave a good fixation in presence of different antigens, but the antitoxic serum had very little complement-fixing property.

In order to demonstrate the presence of specific antibodies, an attempt was made to absorb the group antibody by means of a heterologous antigen of the same group. Fifty to one hundred milligrams of dried and washed bacillary bodies were emulsified in 3 c.c. to 5 c.c. of diluted and inactivated serum. The emulsion was incubated at 37°C. for 1 to 4 hours, shaken at frequent intervals, and later left in the ice-chest. It was centrifuged at high speed next morning. The serum on top was removed and again inactivated at 56°C. for 10 minutes. The absorbed serum was used for the complement-fixation.

It was not found possible in general to absorb the antibody completely by the homologous antigens even. The antigens in use were not easily emulsifiable, hence an intimate antigen-antibody contact could not be established.

In one experiment, *B. subtilis* absorbed the anti-tuberculous antibody in the same degree as tubercle bacilli. It was possible that the antibody absorption had nothing to do with the nature of the antigen, i.e., whether the antigen belonged to the group was immaterial. In his studies (Goyal, 1935) of filter-passers conducted at Edinburgh University, the author had demonstrated antibody absorption by powdered Chamberland filters. Kaolin was utilized for absorption in the present work and it was found that even the specific antibody could be removed by Kaolin. Only one strain belonging to the particular type of tubercle bacilli and described by Saenz *et al.* (1935) showed the presence of a specific antigen, the rest of the strains used in this experiment contained group antigens only. We next attempted to find out if the bacilli far removed from the tubercle bacilli or actinomyces also contained the same group antigens. Rice and Reed (*loc. cit.*) had definitely stated that the

group antigens were found in rough strains, so we utilized the R variants of *B. coli* and *B. subtilis*. In presence of anti-tuberculous sera, 0.5 c.c. of the emulsions containing 1 mg. of bacilli per c.c. either failed to fix complement or caused the fixation of 10 units only. The antisera prepared against these two bacilli fixed 20 to 40 units of homologous antigens and only 0 to 10 units of heterologous methylic antigens. Various other antisera (plague, abortus, anthrax) fixed 0 to 20 units in presence of antigens prepared from acid-fast bacilli or actinomyces. An emulsion of staphylococci fixed only 10 units in presence of anti-tuberculous serum. An anti-staphylococcal serum did not fix tubercle or actinomyces antigens. These results do not authorize us to conclude the presence of an antigen common to all these bacilli and actinomyces-tubercle group.

CONCLUSIONS.

1. The methyl alcohol extract of unemulsifiable strains of actinomyces was found suitable for the complement-fixation test, and was definitely superior to streptothricine.

2. Although a specific antigen was present in certain strains of acid-fast bacilli, yet an antigen common to the actinomyces, corynebacterium, and mycobacterium was clearly demonstrable. Organisms far removed from the tubercle bacilli on a microbiological basis did not possess an appreciable amount of the antigen present in the actinomyces-tubercle group.

3. The mammalian tubercle bacilli and actinomyces were found to have only group antigens, the specific antigens were found to be absent in the methylic extracts.

This work definitely supports the view that the actinomyces should be classified under schizomycetes.

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THE RELATIVE AMOUNTS OF THE PROTEIN AND NON-PROTEIN NITROGENOUS CONSTITUENTS OCCURRING IN FOODSTUFFS AND THEIR SIGNIFICANCE IN THE DETERMINATION OF THE DIGESTIBILITY CO-EFFICIENT OF PROTEINS.

BY

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THE protein content of a foodstuff is commonly estimated by multiplying the total nitrogen by the conventional factor 6.25. On these values are based various data including calculations of calorific value, the digestibility co-efficient, and the biological value of proteins. The practice of multiplying the total nitrogen by 6.25 and expressing the result as protein has been frequently discussed and as frequently criticized (Clark and Almy, 1918; Janney and Gsonka, 1915; Janney, 1916; McCance and Shipp, 1933; McCance, Widdowson and Shackleton, 1936). There are two main objections: first, total nitrogen is not all protein nitrogen, and, secondly, the protein factor 6.25, based on the assumption that protein on an average contains 16 per cent nitrogen, is somewhat arbitrary (Jones, 1931), since values ranging from 12 to 17 have been obtained for different proteins.

Many foodstuffs, e.g., vegetables, contain considerable amounts of non-protein nitrogen (Bhagvat and Sreenivasaya, 1935, 1936; McCance *et al.*, 1933, 1936) part of which may exist as simple derivatives of proteins and amino acids, not precipitated by the majority of the common protein precipitants. Various reagents are employed in the estimation of the protein and non-protein nitrogenous material in foodstuffs and animal tissues, the most important being the following: acetic acid, trichloroacetic acid, tungstic acid, phosphotungstic acid, alcohol, picric acid, metaphosphoric acid, copper hydroxide, and zinc hydroxide.

Van Slyke and Hill (1922) made a comparative study of some of the protein precipitants and showed that they varied widely in the completeness with which they precipitate the intermediate products, such as peptones, peptides, and amino acids. Their conclusions are as follows: 'From the results with Witte's peptone,

it appears that tungstic acid and picric acid are distinguished by the relative completeness with which they precipitate protein intermediate products without precipitating amino acids. Trichloroacetic acid, on the other hand, particularly in solutions more dilute than 5 per cent, permitted nearly all the products to pass into the filtrate. Alcohol behaves towards Witte's peptone like tungstic acid and picric acid but is not a desirable precipitant for quantitative work. Metaphosphoric acid, colloidal iron, and mercuric chloride are intermediate between trichloroacetic acid and tungstic acid in the completeness with which they precipitate the intermediate products of Witte's peptone'.

Similar observations have been made by the author using certain protein precipitants. Table I shows that they differ widely as regards the completeness with which they precipitate the protein intermediate products in red gram: acetic acid gives the highest figure and copper hydroxide the lowest for non-protein nitrogen.

TABLE 1.

The precipitation of the proteins of red gram (Cajanus indicus) by different reagents.

Protein precipitant and method employed.	Protein nitrogen, per cent.	Non-protein nitrogen, per cent.	Non-protein N, expressed as per cent of total N.
Copper hydroxide (Stützer)	3.06	0.24	7.3
Zinc hydroxide (Scott, 1934)	2.96	0.34	10.3
Tungstic acid (Ayres and Lee, 1936)	2.94	0.36	10.9
Trichloroacetic acid (Mezincescu and Szabo, 1936)	2.74	0.56	17.0
Acetic acid (Bhagvat and Sreenivasaya, 1936)	2.52	0.78	23.6

Since peptones, peptides, and amino acids may be regarded as substitutes for pure proteins in nutrition the estimation of the total protein in a foodstuff should include the protein intermediate products. A convenient reagent is found in Stützer's reagent (copper hydroxide); it has been shown by Kober and Sugiura (1913) that even small quantities of amino acids in urine and blood can be quantitatively estimated by using copper hydroxide. Stützer's reagent has been employed in the estimation of protein nitrogen in the present investigation. The non-protein nitrogen was estimated by subtracting the protein nitrogen from the total nitrogen. All the nitrogen estimations were made by the Kjeldahl method. The results obtained are shown in Table II. The samples of food materials for analysis were treated in the manner described in previous communications (Swaminathan, 1937*a* and *c*).

TABLE II.

The percentage of total protein and non-protein nitrogen in foodstuffs.

Name of foodstuff.	Botanical name.	Total nitrogen (T. N.), per cent.	Protein nitrogen (P. N.), per cent.	Non-protein nitrogen (N. P. N.) by difference, per cent.	Non-protein nitrogen, expressed as per cent of total nitrogen.
<i>Cereals.—</i>					
Barley	<i>Hordeum vulgare</i>	1.57	1.46	0.11	7.0
Cambu	<i>Pennisetum typhoideum</i>	2.20	2.05	0.15	6.8
Cholam	<i>Sorghum vulgare</i>	1.36	1.29	0.07	5.1
Italian millet	<i>Setaria italica</i>	1.64	1.53	0.11	6.7
Maize	<i>Zea mays</i>	1.04	1.00	0.04	3.8
Oat meal	<i>Avena sativa</i>	2.11	2.00	0.11	4.1
Ragi	<i>Eleusine coracana</i>	1.13	1.06	0.07	6.2
Rice, raw, milled	<i>Oryza sativa</i>	1.08	1.06	0.02	1.9
Wheat, whole	<i>Triticum vulgare</i>	2.09	1.95	0.14	6.7
<i>Pulses.—</i>					
Bengal gram	<i>Cicer arietinum</i>	3.40	3.30	0.10	2.9
Black gram	<i>Phaseolus mungo</i>	3.61	3.44	0.17	4.7
Cow gram	<i>Vigna catieng</i>	4.10	3.74	0.36	8.8
Field bean, dry	<i>Dolichos lablab</i>	3.65	3.12	0.53	14.5
Green gram	<i>Phaseolus radiatus</i>	3.84	3.68	0.16	4.2
Horse gram	<i>Dolichos biflorus</i>	3.50	3.03	0.47	13.4
Lentil (masur dhal)	<i>Lens esculenta</i>	3.88	3.52	0.36	9.3
Peas, dry	<i>Pisum sativum</i>	3.28	2.98	0.30	9.1
Red gram (dhal arhar)	<i>Cajanus indicus</i>	3.30	3.06	0.24	7.3
Soya bean	<i>Glycine hispida</i>	6.40	5.93	0.47	7.3
<i>Nuts and oilseeds.—</i>					
Almond (de-fatted powder)	<i>Prunus amygdalis</i>	7.96	7.53	0.43	5.4
Cashew-nut (de-fatted powder)	<i>Anacardium occidentale</i>	6.17	5.84	0.33	5.3

TABLE II—*contd.*

Name of foodstuff.	Botanical name.	Total nitrogen (T. N.), per cent.	Protein nitrogen (P. N.), per cent.	Non-protein nitrogen (N. P. N.) by difference, per cent.	Non-protein nitrogen, expressed as per cent of total nitrogen.
<i>Nuts and oilseeds—contd.</i>					
Coco-nut (de-fatted powder) ..	<i>Cocos nucifera</i>	4.11	3.84	0.27	6.6
Gingelly seeds (de-fatted powder)	<i>Sesamum indicum</i>	4.44	4.21	0.23	5.2
Ground-nut " "	<i>Arachis hypogea</i>	6.79	6.50	0.29	4.3
<i>Condiments, spices, etc.—</i>					
' Arisithippli ' " "	<i>Piper clusii</i>	2.37	2.15	0.22	9.3
Coriander " "	<i>Coriandrum sativum</i>	2.28	2.16	0.12	5.3
Cumin .. " "	<i>Cuminum cyminum</i>	2.92	2.74	0.18	6.2
Fenugreek seeds .. "	<i>Trigonella fœnumgræcum</i>	5.46	5.26	0.20	3.7
Ginger, dry, powder "	<i>Zingiber officinale</i>	1.22	1.12	0.10	8.2
Omum (Bishop's weed) .. "	<i>Carum copiticum</i>	1.89	1.81	0.08	4.2
Pepper, dry " "	<i>Piper nigrum</i>	1.97	1.86	0.10	5.6
Turmeric " "	<i>Circuma longa</i>	1.06	1.03	0.03	2.8
<i>Leafy vegetables.—</i>					
Amaranth, tender (dry, powder)	<i>Amaranthus gangeticus</i>	3.59	3.07	0.52	14.5
Coriander (dry, powder) .. "	<i>Coriandrum sativum</i>	3.29	2.88	0.41	12.5
Drumstick " " "	<i>Moringa oleifera</i>	3.58	3.01	0.57	15.9
Ipomœa " " "	<i>Ipomœa reptans</i>	3.79	3.21	0.58	15.3
Lettuce " " "	<i>Lactuca stiva</i>	2.74	2.32	0.42	15.3
Mint " " "	<i>Mentha viridis</i>	3.53	3.00	0.53	15.0
Sesbania " " "	<i>Sesbania grandiflora</i>	4.81	4.04	0.77	16.0
Spinach " " "	<i>Spinacia oleracea</i>	3.93	3.51	0.42	10.7

TABLE II—concl'd.

Name of foodstuff.	Botanical name.	Total nitrogen (T. N.), per cent.	Protein nitrogen (P. N.), per cent.	Non-protein nitrogen (N. P. N.) by difference, per cent.	Non-protein nitrogen, expressed as per cent of total nitrogen.
<i>Roots and tubers.—</i>					
Carrot (dry, powder) ..	<i>Daucus carota</i>	1.02	0.93	0.09	8.8
Potato „ „ ..	<i>Solanum tuberosum</i>	1.06	0.74	0.32	30.2
Radish (pink) „ ..	<i>Raphanus sativus</i>	0.98	0.91	0.07	7.1
Tapioca „ „ ..	<i>Manihot utilissima</i>	0.25	0.22	0.03	12.0
<i>Other vegetables.—</i>					
Bitter gourd (dry, powder) ..	<i>Momordica charantia</i>	2.17	1.97	0.20	9.2
Brinjal „ „ ..	<i>Solanum melongena</i>	2.23	1.96	0.27	12.1
Double beans „ „ ..	<i>Faba vulgaris</i>	4.13	3.58	0.55	13.3
Jack-fruit seeds „ „ ..	<i>Artocarpus integrifolia</i>	1.70	1.55	0.15	8.8
Lady's fingers „ „ ..	<i>Hibiscus esculentus</i>	2.10	1.75	0.35	16.7
Peas, green „ „ ..	<i>Pisum sativum</i>	3.99	3.45	0.54	13.5
Plantain, green „ „ ..	<i>Musa paradisiaca</i>	0.69	0.61	0.08	11.6
<i>Milk.—</i>					
Milk, buffalo's	0.69	0.61	0.08	11.6
Milk, cow's	0.59	0.55	0.04	6.8
Milk, goat's	0.59	0.55	0.04	6.8
Milk, human	0.16	0.14	0.02	12.5
Skimmed milk powder	6.61	6.23	0.38	5.7

DISCUSSION.

Although the exact rôle played in protein metabolism by the non-protein nitrogenous constituents of foodstuffs is at present unknown, it is of interest to note that the foodstuffs investigated vary considerably in their non-protein nitrogen content ; on an average cereals contain 5 per cent, pulses 9 per cent, nuts and oilseeds 5 per cent, condiments 6 per cent, vegetables 14 per cent, and milk 9 per cent of their total nitrogen in the form of non-protein nitrogen.

Very little is known regarding the nature of the non-protein nitrogenous constituents not precipitated by copper hydroxide. Hamilton *et al.* (1921) found that about 2 per cent of the total nitrogen of oats and corn are soluble in absolute ether and alcohol, while Horwitt *et al.* (1936) showed that about 6 per cent of the total nitrogen in spinach is ether soluble. McCance, Widdowson and Shackleton (*loc. cit.*) have investigated the nature of the nitrogenous material in certain vegetables and nuts. Their observations are summarized as follows:—

‘In 36 representative fruits, vegetables, and nuts, the amount of nitrogen soluble in 90 per cent alcohol has been determined as well as the total N. It will be seen that all the nitrogen in nuts is insoluble and therefore may be considered protein nitrogen. In fruits and vegetables, however, about one-third of the nitrogen is soluble in 90 per cent alcohol. It is improbable that this is protein N and in most cases little is known of the manner in which this nitrogen is combined. Bhagvat and Sreenivasaya (1935) have stated that the non-protein N of the pulses consists of easily digestible and assimilable peptides. If this is true of other vegetables and fruits, it is perhaps fair to assume that almost all their nitrogen is amino-acid nitrogen, if not protein nitrogen, and the use of the factor 6.25 is legitimate. Mushrooms, however, are known to contain large amounts of urea and it is clear, therefore, that it is incorrect to multiply the total N of this vegetable by 6.25 and state the result as protein’.

THE NON-PROTEIN NITROGEN AND ITS SIGNIFICANCE IN THE ESTIMATION OF THE DIGESTIBILITY CO-EFFICIENT OF PROTEINS.

In the determination of the digestibility co-efficient and the biological value of proteins, the total nitrogen content of a foodstuff is taken into account (Mitchell, 1924*a* and *b*; Mitchell *et al.*, 1924, 1926; Boas-Fixsen *et al.*, 1932, 1934; Chick *et al.*, 1935*a* and *b*; Swaminathan, 1937*a*, *b*, *c* and *d*).

The co-efficients of digestibility and the biological values determined in such investigations, although referred to in terms of the conventional ‘protein’ ($N \times 6.25$), are in reality based on the total nitrogen content. The wastage of protein in digestion seems to be largely unrelated to its chemical structure, though the biological value of a protein is generally ascribed entirely to its chemical structure (Mitchell, 1924*a*).

Mendel and Fine (1911, 1912) have shown that the differences in digestibility between different proteins largely disappear when vegetable proteins are fed in a nearly pure condition. They found that the proteins of wheat, barley, and corn in a pure state were as well utilized as the proteins of meat. They explained the low digestibility of the proteins of soya bean, navy bean, and peas on the grounds that these proteins are less readily hydrolysed by the digestive process than the cereal proteins. It is quite likely that the non-protein nitrogenous materials (not precipitated by copper hydroxide) occurring in foodstuffs are not fully digested and hence provide the main contribution to the loss of nitrogen in digestion. Thus it is probable that the digestibility co-efficients obtained for the ‘crude protein’ in foodstuffs represent an under-estimation because of the presence of non-protein nitrogenous constituents, non-amino and non-peptide in character. The loss of food nitrogen in digestion calculated from previous experiments on rats (Swaminathan, *loc. cit.*) and the non-protein nitrogen content of foodstuffs are

shown in Table III. It will be seen that the pulses and vegetables, whose proteins have a low digestibility co-efficient, are rich in non-protein nitrogen.

TABLE III.

The loss of food nitrogen in digestion and the non-protein nitrogen content of foodstuffs (expressed as per cent of the total nitrogen).

Name of foodstuff.			Loss of food nitrogen in digestion, per cent.	Non-protein nitrogen, per cent.
<i>Cereals.—</i>				
Cambu	11	7
Cholam	9	5
Italian millet	9	7
Ragi	20	6
Rice, raw, milled	3	2
Wheat, whole	7	7
<i>Pulses.—</i>				
Bengal gram	14	3
Black gram	22	5
Cow gram	22	9
Field bean, dry	24	15
Green gram	14	4
Horse gram	27	13
Lentil	12	9
Red gram	25	7
Soya bean	24	7
<i>Nuts and oilseeds.—</i>				
Cashew-nut	10	5
Coco-nut	6	6
Gingelly seeds	15	5
Ground-nut	10	4

TABLE III—*concl'd.*

Name of food-stuff.	Loss of food nitrogen in digestion, per cent.	Non-protein nitrogen, per cent.
<i>Milk.</i> —		
Skimmed milk powder ..	10	6
<i>Vegetables.</i> —		
Amaranth leaves, tender ..	22	15
Drumstick leaves ..	23	16
Ipomœa leaves	15	15
Sesbania leaves	15	15
Brinjal	25	12
Lady's fingers .. .	30	17

CONCLUSION.

The method of estimating the protein content of a foodstuff, by multiplying the total nitrogen content by the factor 6.25, is not fully justified since appreciable amounts of non-protein nitrogen, non-amino and non-peptide in character, form part of the total nitrogen; considered in terms of practical dietetics, however, the error involved is not of great importance.

SUMMARY.

1. The relative amounts of protein and non-protein nitrogenous constituents occurring in certain cereals, pulses, nuts, oilseeds, condiments, vegetables, and milk have been determined by the Stützer method.

2. The average amounts of non-protein nitrogen, expressed as per cent of total nitrogen, occurring in the different groups of foodstuffs, were as follows: cereals 5 per cent; pulses 9 per cent; nuts and oilseeds 5 per cent; condiments 6 per cent; vegetables 14 per cent; and milk 9 per cent.

3. If the protein content of a food is determined by multiplying the nitrogen content by 6.25 the digestibility co-efficient of 'proteins' may be under-estimated, owing to the presence of varying amounts of non-protein nitrogen which may be poorly available in digestion.

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FURTHER STUDIES ON FACTORS AFFECTING THE VITAMIN-A ACTIVITY OF ANIMAL AND VEGETABLE PRODUCTS.

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IN continuation of work reported in a previous communication (De, 1936), further experiments were carried out to study the effect of heat, cooking, æration, hydrogenation, etc., on the vitamin-A and carotene content of certain foodstuffs of both animal and vegetable origin. It is a popularly known fact that 'dhals' (legumes) can be quickly cooked, if boiling takes place in an alkaline medium containing a little sodium bicarbonate. Certain foods, on the other hand, are sometimes cooked in an acid medium, with an addition of a little tamarind. The effect of cooking with sodium bicarbonate and tamarind on vitamin-A and carotene content has also been studied.

From the literature it appears that only biological methods have been applied in almost all the work which has so far been carried out on this subject. While there appears to be substantial agreement that the vitamin-A activity of vegetable products is little affected by cooking and canning (Steenbock and Boutwell, 1920 ; Mendel, 1932 ; Sure, 1933 ; Coward and Morgan, 1935 ; and others) results obtained with animal materials, especially butter and ghee, are less clear-cut (Osborne and Mendel, 1920 ; Hopkins, 1920 ; Zilva, 1920 ; Drummond and Coward, 1920 ; Hess, 1920 ; Banerjee and Dastur, 1937 ; and others). It has, however, been proved beyond doubt that heat in the presence of air or oxygen, æration, and even hydrogenation are destructive of vitamin A (Grewal, 1935 ; Drummond, Cannon and Coward, 1925 ; and others). A loss of vitamin A has been observed in milk as a result of boiling (Decaro and Speier, 1935 ; and others).

In earlier work on this subject estimations of vitamin A were made by feeding experiments, and most of the results were expressed in qualitative terms. Since it is important to have accurate quantitative data on the question, an investigation was undertaken using spectrophotometric methods of assaying vitamin A and pro-vitamin A (carotene). The principle and the details of the method adopted are similar to those described previously (De, 1937). The results are set out in the following tables :—

TABLE I.

Effect of cooking, etc., on the carotene content of some vegetable products.

Experi- ment number.	Name of the food and description of the treatment applied.	Carotene content per gramme of original material (γ).
1	Amaranth (<i>Amaranthus gangeticus</i>), fresh raw sample ..	108.0
	„ cooked (1 g. in 30 c.c. water, for about 20 minutes).	110.0
	„ „ (1 g. in 30 c.c. water, containing 250 mg. of common salt, for about 20 minutes).	107.0
	„ „ (1 g. in 30 c.c. water, containing 20 mg. of NaHCO_3 , for about 15 minutes).	109.0
	„ „ (1 g. in 30 c.c. water—250 mg. of tamarind, for about 40 minutes).	104.0
2	Carrot (<i>Daucus carota</i>), cooked for about 45 minutes ..	43.2
	„ cooked with NaHCO_3 , for about 20 minutes ..	42.8
	„ „ „ tamarind, for about 45 minutes ..	48.0
3	Amaranth (fresh raw sample)	87.0
	„ fried in coco-nut oil	86.0
4	Carrot (fresh raw)	74.5
	„ fried in coco-nut oil	72.0
5	Green gram (<i>Phaseolus radiatus</i>), raw	2.9
	„ cooked (5 g. gram with 1 g. tamarind, for about 45 minutes).	3.1
	„ cooked (5 g. gram with 200 mg. NaHCO_3 , for about 15 minutes).	3.0
6	Red gram (control)	2.6

TABLE I--*concl'd.*

Experiment number.	Name of the food and description of the treatment applied.	Carotene content per gramme of original material (γ).
	Red gram cooked (5 g. gram with 1 g. tamarind, for about 45 minutes).	2.4
	„ cooked (5 g. gram with 200 mg. NaHCO_3 , for about 20 minutes).	2.7
7	Red-palm oil (<i>Elæis guineensis</i>), uncooked	400.0
	„ „ aerated for four hours at 90°C. to 100°C. ..	210.0 loss of carotene 47.5 per cent.
	„ „ hydrogenated for four hours at 90°C. to 100°C.	270.0 loss of carotene 32.5 per cent.

TABLE II.

Effect of cooking, etc., on the vitamin-A content of some animal products.

Experiment number.	Name of the material and description of the treatment applied.	Vitamin-A content, per gramme (γ).	Vitamin content of treated material, per cent of that of untreated sample.
1	Egg yolk (raw)	3.8 (Carotene 17.0 γ /g.)	..
	Egg yolk after heating for one hour (the egg was placed in a test-tube and immersed in boiling water).	4.0 (Carotene 18.0 γ /g.)	..
2	Sheep liver (raw)	103.7	..
	„ „ cooked with tamarind until soft	105.2	..
	„ „ cooked with NaHCO_3 until soft	102.1	..
3	Butter (uncooked)	11.25	..
	Ghee prepared from above butter by heating for 45 minutes from cold to maximum temperature of 150°C. Loss of weight due to evaporation of moisture, etc. = 20.5 per cent.	8.75	77.8

TABLE II—*contd.*

Experi- ment number.	Name of the material and description of the treatment applied.	Vitamin-A content, per gramme (γ).	Vitamin content of treated material, per cent of that of untreated sample.
4	Butter (uncooked)	10.0	..
	Ghee made from above butter heated from cold for 22 minutes, maximum temperature 120°C. Loss of weight due to evaporation of moisture = 19.4 per cent.	8.95	89.5
	Ghee heated directly for half an hour to a maximum temperature of about 270°C.	Nil or trace	..
	Above butter heated on a water-bath in an open vessel for six hours (5 g. in a 250-c.c. basin).	Nil or trace	..
	Above ghee heated on a water-bath in an open vessel for six hours (5 g. in a 250-c.c. basin).	Nil or trace	..
5	Butter (uncooked)	6.8	..
	Ghee prepared from above butter by heating from cold for 30 minutes, maximum temperature 130°C.	5.6	82.3
	Butter aerated for six hours at 100°C. (125 g. in 250-c.c. flask, two to three bubbles of air per second).	2.5	36.8
	Above butter heated on a water-bath for six hours (50 g. in 250-c.c. basin).	2.9	42.7
	Above ghee, after being used for frying potato chips.	4.8	70.3
	Above ghee after 2nd frying ..	3.1	45.6
	Potato chips fried in the 1st ..	2.0	..
6	Butter (uncooked)	5.62	..
	Butter 125 g. aerated for six hours at 100°C., two to three bubbles of air per second.	2.96	52.7
	Above butter preserved at 0°C. for two weeks.	5.6	100.0
	Above butter preserved at room temperature (15°C. to 22°C.) for two weeks.	4.4	78.3
	Above butter preserved at 37°C. for two weeks.	3.0	53.4

TABLE II—*concl'd.*

Experi- ment number.	Name of the material and description of the treatment applied.	Vitamin-A content, per gramme (γ).	Vitamin content of treated material, per cent of that of untreated sample.
7	Cod-liver oil (raw)	500.0	..
	Cod-liver oil heated on water-bath for four hours in an open vessel, tempera- ture 95°C. to 100°C.	170.0	34.0
	Cod-liver oil hydrogenated for four hours at 95°C. to 100°C.	265.0	53.0
8	Milk (uncooked)	0.50	..
	„ just boiled	0.52 per g. of original material.	104.0
	„ concentrated by prolonged boiling	0.32 „	64.0
	Curds prepared from the same milk by adding citric acid and keeping for 24 hours at room temperature.	0.49 „	98.0
9	Milk (different sample)	0.30 „	..
	Milk, just boiled	0.28 „	93.3
	Milk, concentrated by prolonged boiling	0.19 „	63.3
10	Milk (different sample)	0.56 „	..
	Curds (sour milk) prepared from the same milk with lactic acid bacilli and fermenting for 48 hours at room tem- perature.	0.50 „	89.3
	Curds (sour milk) prepared from the same milk with lactic acid bacilli and fermenting for 48 hours at 37°C.	0.38 „	67.9

Note.—Losses of carotene in butter and milk were not studied because butter and milk contain carotene only in very small quantities.

DISCUSSION.

It is well known that vitamin A, including its precursors, can readily be destroyed by oxygen or oxidizing agents at high temperatures. Nevertheless vitamin A protected by the chemical 'environment' in which it exists in most of its natural sources is somewhat more resistant to destruction than was formerly assumed. Recent researches (Mendel, *loc. cit.*) have proved that vitamin A (including its precursors) possesses sufficient stability, as it exists in most common foods, to undergo the modern processes of food conservation. It is, however, evident that

destruction of vitamin A or carotene is more marked when the potent factors are separated from the products with which they are associated in nature.

In general vitamin A and carotene survive with only a small loss of potency the culinary processes of civilized living. It appears that there has been an increasing tendency in France and in England to use domestic autoclaves for cooking food; this practice must be detrimental to the vitamins A and C contained in food materials, since the temperature in the autoclaves reaches 140°C. or more.

SUMMARY.

1. In general the vitamin-A and carotene content of foodstuffs is not affected to any appreciable extent by boiling in water in open vessels.

2. The use of sodium bicarbonate and tamarind in cooking has no destructive influence on vitamin A and carotene. Sodium bicarbonate hastens the process of cooking.

3. Appreciable loss (10 to 22 per cent) of vitamin A occurs during the melting of butter into ghee, the amount of loss depending on the degree of heat applied.

4. Both æration and hydrogenation, and also heating on a water-bath in open vessels, were found to cause considerable destruction of the vitamin A contained in butter, ghee, cod-liver oil, and red-palm oil.

5. Ghee used in frying potato chips loses a considerable portion of its vitamin-A content during each operation of frying.

6. Prolonged heating causes a marked loss of vitamin A from milk.

7. Sour whole milk was found to be poorer in vitamin A than the original milk from which it was prepared; the loss was more marked when the curd was prepared by fermenting milk at 37°C. than at room temperature (16°C. to 22°C.).

8. No loss of vitamin A occurred in butter preserved for two weeks at 0°C.: a definite loss was noted in the case of samples kept for the same period at room temperature and in the incubator respectively.

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THE NITROGEN COMPLEX OF INDIAN FOODSTUFFS.

CONDIMENTS : PART II. CHILLIES (*CAPSICUM ANNUM*) AND CORIANDER SEEDS (*CORIANDRUM SATIVUM*).

BY

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In a previous communication (Narasimhamurthy and Ranganathan, 1937), the results of investigations of the nitrogen complex of black pepper (*Piper nigrum*) and its value in nutrition were presented. Pepper is used as a condiment and is held to possess carminative properties. Chillies and coriander belong to the same group but are included in the Indian dietary in more liberal quantities than pepper. The present paper describes a study of the nitrogen complex of these condiments.

The cultivation of chillies is largely confined to Madras, Bengal, Bombay, and the Punjab, the first mentioned being the largest producer. Official statistics show that, in the Madras Presidency, the acreage under chillies during 1934-35 was 453,094 acres, an area roughly 43 per cent of the total area devoted to the cultivation of condiments. The most commonly grown variety is the long and seedy one (*Capsicum annum*). The crop is harvested when the fruits are ripe and scarlet; the fruits are dried in the sun before consumption. Chillies are also not uncommonly eaten in the raw green state; in this condition the foodstuff is a useful source of vitamin C. The extent of its use varies from place to place and no exact figure can be given. In the Northern Circars, where it is grown and consumed more extensively than in the rest of the country, its consumption may be as high as 12 to 15 grammes per head per day.

Coriander is a minor condiment crop compared with chillies but is more extensively grown than pepper. In the Madras Presidency in the year 1934-35 it occupied 17 per cent of the total land under condiments, whereas pepper covered about 14 per cent. The crop is consumed when the fruits are ripe and dry. The extent of its consumption is variable as in the case of chillies but in general much smaller amounts are used in cooking.

EXPERIMENTAL.

The foodstuffs were milled to pass through a 50-mesh sieve. Chemical analysis gave the following figures:—

Chemical composition of chillies and coriander seeds.

	Moisture.	Nitrogen.	Fat.	Fibre.	Ash.	Calcium.	Phosphorus.	Iron.	Carbo-hydrates by difference.
Chillies ..	8.6	2.35	6.24	30.15	6.1	0.16	0.37	2.25	34.23
Coriander	6.1	2.37	9.8	32.58	4.39	0.63	0.36	17.94	32.32

Two series of experiments on the nitrogen complex were carried out. In one series, the pH levels and the temperature of the extracting media resembled those present in the alimentary canal: in the other, the temperature and the concentration of salt used in cooking were simulated. In the first series, one part of the test material was extracted with 10 parts of the extracting media (water with varying quantities of salt) at different levels of pH for one hour at 37°C. In the second, 40 parts of the extracting media were employed, and the operation was carried out for one hour, or half an hour, at 100°C. at two levels of pH. The dilution and salt concentration in this series corresponded to those found in two common preparations, 'rasam' and 'sambar', which are courses in the South Indian meal. 'Rasam' is a dilute water extract of tamarind, salt, and chilly powder to which pepper powder is usually added. 'Sambar' is a similar preparation containing dhal and vegetables; it is much thicker than rasam, corresponding in consistency to thick soup. Coriander seeds may be added to these preparations.

TABLE I.

Influence of pH and salt concentration on the extractable nitrogen of chillies and coriander seeds extracted for various periods (quantities expressed as percentages of the ground material).

Salt (parts per cent), pH.	Quantity of nitrogen extracted from chillies.				Quantity of nitrogen extracted from coriander seeds.			
	0	1	2	3	0	1	2	3
<i>Extracted at 37°C. for one hour.</i>								
1.0	1.15	1.06	1.16	1.12	0.34	0.34	0.32	0.30
3.0	1.08	1.01	1.24	1.14	0.30	0.29	0.29	0.32
5.0	1.08	1.17	1.27	1.33	0.34	0.34	0.39	0.38
7.0	1.21	1.22	1.23	1.35	0.57	0.50	0.55	0.57
9.0	1.41	1.47	1.41	1.39	0.61	0.72	0.60	0.57

TABLE I—*conold.*

Salt (parts per cent), pH.	Quantity of nitrogen extracted from chillies.				Quantity of nitrogen extracted from coriander seeds.			
	0	1	2	3	0	1	2	3
<i>Extracted at 100°C. for one hour.</i>								
1.0	1.78	1.16	1.36	1.25	1.69	1.18	0.99	0.81
3.0	1.07	1.10	1.14	1.07	0.42	0.53	0.50	0.53
<i>Extracted at 100°C. for half an hour.</i>								
1.0	1.67	1.32	1.18	1.16	1.56	0.68	0.70	0.70
3.0	1.10	1.07	1.10	1.10	0.44	0.61	0.46	0.42

The forms in which the nitrogen exists in these materials were also investigated. Albumins (in which is included the non-protein nitrogen), globulins, prolamins, and glutelins were determined. Details about the experimental procedure and the reagents used were given in the earlier publication (Narasimhamurthy and Ranganathan, *loc. cit.*). The results are summarized in Table II:—

TABLE II.

Protein fractions in chillies and coriander seeds.

Nitrogen, per cent as:—	Albumins.	Globulins.	Prolamins.	Glutelins.	Non-extractable N.
Chillies ..	1.06	0.46	0.13	0.44	0.26
Coriander ..	0.48	0.06	0.02	0.15	1.66

The non-protein nitrogen of these two foodstuffs was also determined by three procedures. The results are shown in Table III:—

TABLE III.

Non-protein nitrogen of chillies and coriander seeds expressed as per cent of original material.

Chillies ..	0.74	0.71	0.74
Coriander ..	0.23	0.20	0.23
	Method of Merincescu and Szabo (1936).	Method of Ayres and Lee (1936).	Trichloro-acetic acid (2.0 per cent).

The final series of experiments was carried out on samples rendered free from fat, the nitrogen distribution being studied by the method of van Slyke (1911) as modified by Plimmer and Rosedale (1925). Arginine and cystine were estimated in the basic fraction. Tryptophane and tyrosine were estimated separately on the fat-free material colorimetrically by the method of Folin and Meranzi (1929). In Table IV the results are summarized :—

TABLE IV.
Nitrogen distribution in chillies and coriander (expressed as percentages of the total nitrogen).

	Chillies.	Coriander.
Humin (Melanin)	10.643	7.563
Amide	11.110	14.042
<i>Basic, comprising :—</i>		
Arginine	0.907	7.711
Histidine	0.975	1.256
Cystine	<i>Nil</i>	Traces
Lysine	10.998	3.483
<i>Non-basic, comprising :—</i>		
Amino	52.710	5.234
Non-amino	12.060	59.791
TOTAL ..	99.403	99.080
<i>Amino acids estimated on the fat-free material :—</i>		
Tyrosine (direct estimation) ..	0.924	1.890
Tryptophane „ „ ..	0.262	0.372

DISCUSSION.

In the case of chillies, the major portion of the nitrogen is extracted under conditions resembling those employed in cooking and those occurring in digestion. Of this 'available' nitrogen over 40 per cent is in the 'non-protein' form. Under similar conditions of temperature, pH, and salt concentration, coriander seeds do not part with a corresponding proportion of their nitrogen. Chillies are superior to coriander seeds in their content of readily available forms of protein, e.g., albumin and globulin.

Cystine is not present, or is present in very small amount, in both foodstuffs. Its absence from the basic fraction raises the figure for lysine, which is calculated by difference. Tryptophane and histidine, two of the amino acids which are of importance in nutrition, are present in small quantities. It thus appears that the amino acid composition of these condiments is not such as to make them valuable as supplements to diets containing insufficient protein of high biological value.

SUMMARY.

1. Chillies and coriander seeds were analysed for the available protein and non-protein fractions of their nitrogen complex. The nitrogen distribution in the nitrogen complex was studied by the van Slyke technique. The proportion of extractable nitrogen in the former was greater than in the latter.

2. Cystine was found to be absent from both foodstuffs, while tryptophane and histidine were present in very small amounts.

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THE AVAILABILITY OF PHOSPHORUS FROM INDIAN FOODSTUFFS.

BY

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THE mineral requirements of man have received considerable attention during recent years, and interest has mainly been confined to the study of calcium, phosphorus, and iron requirements. A deficiency in the intake of these minerals brings about certain types of diseases like rickets, anæmia, general weakness, and other pathological derangements.

It is now being recognized that the degree of absorption of these constituents into the body is dependent on the form in which they are supplied. The mineral salts present in foodstuffs may be unavailable if they are in some form which cannot be absorbed. A careful survey of foodstuffs generally consumed by people, with respect to their availability of minerals, is therefore the most important item in nutrition.

Ever since the discovery by E. Mellanby (1918) and M. Mellanby (1918) that high cereal diet causes faulty skeletal development and poor teeth in dogs and rats, attention was directed by workers on nutrition to the study of the agent responsible for the poor calcifying properties of cereal grains. Later work led E. Mellanby (1926) to suggest the existence in cereal grains of some distinct rachitogenic substances or anti-vitamins, which he called toxamins. Further researches have definitely established the anti-calcifying effect of cereals on teeth (M. Mellanby, 1928, 1929; King and Hall, 1931). Later workers have tried to ascribe the rachitogenic effect of cereals to factors other than toxamins. Thus, Fine (1930) showed that the difference between cereals with regard to their rachitogenic properties is due to the difference in vitamin-D content, while Bruce and Callow (1934) state 'the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that cereal phosphorus is not in an available form', and that the inferiority of oat meal is due to the lower availability of its phosphorus,

present chiefly as phytin. The fate of ingested phytin in the human body was investigated by McCance and Widdowson (1935), who found that 20 to 60 per cent of the phytin was excreted unchanged in the faeces. Recently, Lowe and Steenbock (1936) have definitely established that phytin is a poorly available source of phosphorus when fed to the rat in contrast with phosphoric acid and sodium glycerophosphate.

Phosphorus is present in foodstuffs in part in the form of phytin (calcium-magnesium salt of inositol hexaphosphoric acid). Since it is now generally agreed that phytin phosphorus in foods is not available for nutrition, because it passes through the intestine unchanged, and does not serve as a source of phosphorus to the body, it is clear that a knowledge of the total phosphorus in a foodstuff is of less value from a dietetic standpoint. A knowledge of the non-phytin phosphorus is probably of much more value although it cannot be assumed that the whole of this phosphorus will be available or that the whole of the phytin phosphorus will be unavailable. Further, in view of the suggestion made by Lowe and Steenbock (*loc. cit.*) that 'in considering the mode of hydrolysis of phytin in the intestine it is obvious that the phytase of the flora of the gastro-intestinal tract as well as the phytase ingested with the food warrant intensive investigation', it is apparent that unquestionably data on the phytase activities of foodstuffs should be obtained. The object of the present investigation was to present and interpret data from the analysis of a large number of typical Indian foods, with respect to their total phosphorus, phytin phosphorus, and phytase activity.

EXPERIMENTAL.

All the food materials used in the present study were obtained fresh from the market. Care was taken to select only fresh and representative specimens. Immediately upon reaching the laboratory the plant materials were prepared for analysis. The edible portion of the plants were separated from the non-edible, which was discarded. A small part of the fresh edible portion of each specimen was taken for the moisture determination and the remainder was dried at 45°C. to 50°C. Some materials which tended to become mouldy, when dried at low temperature, were heated in an oven at a temperature of about 80°C. to 90°C. until sufficiently dry. All the samples when dried were ground to a fine powder, and kept in well-stoppered bottles.

The foodstuffs were analysed for moisture, total P, phytin P, and phytase activity.

Moisture.—The moisture content was determined by heating a portion of the sample to constant weight in an electric oven at a temperature of about 105°C.

Total phosphorus.—The dried material (dried at 50°C.) was digested with perchloric-sulphuric acid mixture as recommended by King (1932). The mixture, after neutralization, was made up to a known volume and the inorganic P was determined colorimetrically by the method of Fiske and Subbarow (1925).

Phytin phosphorus.—Determinations of phytin phosphorus were made according to McCance and Widdowson's (*loc. cit.*) method.

Phytase. The preparation of materials for extraction of the enzyme.—The cereals were powdered well and passed through a 60-mesh sieve. Tubers and green vegetables were ground fresh in a mincing machine and the shredded pulp was immediately used for extraction. Fresh leafy vegetables were similarly treated, and the shredded pulp was directly used for extraction.

Preparation of active extracts.—Ten-gramme lots of the powdered cereals, oil seeds, and nuts were extracted with 50 c.c. of toluenated water for 24 hours at room temperature (26°C. to 29°C.) and filtered. The filtrate was directly used for the determination of the activity. In the case of seeds (soya bean) which contain high concentrations of the enzyme, the volume of the water used for extraction was proportionately increased. The same procedure was adopted for the preparation of active extracts from vegetables, tubers, and leaves.

Determination of phytase activity.—The activity of the phytase was determined at pH 5.2 and at $35^{\circ} \pm 0.1^{\circ}\text{C}$. The reaction mixture contained 10 mg. of phytin phosphorus. Sodium salt of inositol hexaphosphoric acid was used as substrate. Five c.c. of the aqueous extract were used for activity determination. The reaction was allowed to proceed for 6 hours and the inorganic phosphorus released was determined in 10 c.c. of the reaction mixture. From the value thus obtained the activity of the phytase contained in the total volume of the extract corresponding to 1 gramme of dry material was calculated. The figures given in the Table represent the relative activities of the phytase contained in the aqueous extract, which correspond to 1 gramme of the moisture-free material.

Preparation of the sodium salt of inositol hexaphosphoric acid.—The salt was prepared by the method of Posternak (1919) with slight modification.

Five grammes of phytin (Kahlbaum) were dissolved in 20 c.c. of N/2 hydrochloric acid, and ferric chloride solution was added until a persisting yellow colour was formed. The ferric salt of inositol hexaphosphoric acid was precipitated, while the inorganic iron phosphate remained dissolved in the acid solution. The ferric salt was removed by filtration, washed repeatedly with water, and a homogeneous suspension of it was made in water. Pure normal sodium hydroxide solution was slowly added to the suspension of the ferric salt under constant stirring, until a drop of the mixture, when placed on a filter-paper, formed a brown spot with a colourless edge. It was then filtered and washed. To the filtrate was added half its volume of alcohol, and kept in an ice-chest overnight. A syrupy crystalline mass separated at the bottom of the flask. The alcohol was removed by decantation and the crystalline viscous mass was again dissolved in water and heated on a water-bath, in order to drive off traces of alcohol and excess of water. On cooling the sodium salt of inositol hexaphosphoric acid crystallized out. The pure salt was dissolved in water, and used after suitable dilution as substrate for the phytase activity determination. The solution was diluted to contain 10 mg. of total P in 5 c.c.

TABLE.

Number.	Name of foodstuff.	Botanical name.	Moisture, per cent.	Total P, per cent.	Phytin P, per cent.	Phytin P as per cent of total P.	' Available ' non-phytin P as per cent of total P.	Phytase.
<i>Cereals.</i> —								
1	Barley ..	<i>Hordeum vulgare</i>	14.1	0.284	0.163	57.4	42.3	0.091
2	Cambu ..	<i>Pennisetum typhoides</i>	10.86	0.230	0.114	49.6	50.4	0.174
3	Cholam ..	<i>Sorghum vulgare</i>	11.77	0.374	0.228	60.2	39.8	0.076
4	Maize ..	<i>Zea mays</i>						
	(a) Milky stage ..	"	67.7	0.393	0.107	27.2	72.8	0.133
	(b) Fully set grain ..	"	47.0	0.324	0.224	69.1	30.9	0.063
5	Ragi ..	<i>Eleusine coracana</i>	11.85	0.245	0.172	70.2	29.8	0.010
	" cooked ..	"	65.2	0.244	0.150	61.4	38.6	..
6	Rice (Mysore) ..	<i>Oryza sativa</i>	12.7	0.354	0.204	57.7	42.3	0.029
	" parboiled ..	"	12.5	0.391	0.210	53.7	46.3	..
	" (G. E. B. 24) unpolished ..	"	10.8	0.283	0.156	54.6	45.4	..
	" polished (14 per cent) ..	"	11.1	0.118	0.027	22.9	77.1	..
7	Wheat ..	<i>Triticum vulgare</i>						
	(a) whole wheat ..	"	12.47	0.229	0.138	60.3	39.7	0.120
	(b) polished (11 per cent) ..	"	13.00	0.130	0.078	60.0	40.0	..
	(c) polished (25 ..) ..	"	12.7	0.101	0.038	47.5	52.5	..

<i>Pulses.</i> —										
8	Bengal gram	<i>Cicer arietinum</i>	10.73	0.371	0.223	60.1	30.9	0.073
	"	soaked in water	..	"	13.88	0.390	0.135	39.7	60.3	..
	for 20 hours.									
9	Black gram	<i>Phaseolus mungo</i>	11.22	0.301	0.123	40.9	59.1	0.073
	"	soaked in water	..	"	13.94	0.315	0.0688	21.8	78.2	..
	for 24 hours.									
10	Cow pea	<i>Vigna catiung</i>	11.8	0.519	0.192	35.0	65.0	0.158
	"	soaked in water	..	"	12.1	0.566	0.178	31.4	68.6	..
	for 24 hours.									
11	Field bean	<i>Dolichos lablab</i>	12.1	0.382	0.119	31.2	68.9	0.182
12	Green gram	<i>Phaseolus radiatus</i>	13.28	0.409	0.208	50.8	49.2	0.210
	"	soaked in water	..	"	15.04	0.420	0.106	25.2	74.8	..
	for 20 hours.									
13	Horse gram	<i>Dolichos biflorus</i>	11.1	0.275	0.048	17.5	82.5	0.127
14	Peas (dried)	<i>Pisum sativum</i>	12.5	0.476	0.147	30.9	69.1	0.162
	" (roasted)	"	8.48	0.440	0.139	31.6	68.4	..
15	Red gram	<i>Cajanus indicus</i>	10.75	0.330	0.139	42.1	57.9	0.134
16	Soya bean	<i>Glycine hispida</i>	10.0	0.832	0.278	33.4	66.6	0.110
	<i>Oil seeds and nuts.</i> —									
17	Almonds	<i>Prunus amygdalis</i>	6.16	0.645	0.242	37.5	62.5	0.095
18	Brazil nut	<i>Bertholletia excelsa</i>	4.0	0.805	0.313	37.6	62.4	0.106
19	Cashew-nut	<i>Anacardium occidentale</i>	6.5	0.574	0.170	29.6	70.4	0.105
20	Coco-nut	<i>Cocos nucifera</i>	4.76	0.209	0.114	54.5	45.5	0.046

TABLE—*concl'd.*

Number.	Name of foodstuff.	Botanical name.	Moisture, per cent.	Total P, per cent.	Phytin P, per cent.	Phytin P as per cent of total P.	'Available', non-phytin P as per cent of total P.	Phytase.
<i>Oil seeds and nuts—concl'd.</i>								
21	Gingelly ..	<i>Sesamum indicum</i>	5.5	0.569	0.160	28.1	71.9	0.169
22	Ground-nut ..	<i>Arachis hypogaea</i>	5.6	0.312	0.133	42.6	57.4	0.074
23	Mustard ..	<i>Brassica juncea</i>	8.1	0.651	0.178	27.3	72.7	0.154
24	Walnut ..	<i>Juglans regia</i>	4.61	0.403	0.179	44.4	55.6	0.100
<i>Roots and tubers.—</i>								
25	Carrots ..	<i>Daucus carota</i>	91.5	0.042	0.0016	3.8	96.2	1.16
26	Potato ..	<i>Solanum tuberosum</i>	78.0	0.047	0.0053	11.3	88.7	0.125
27	Radish (white) ..	<i>Raphanus sativus</i>	96.7	0.028	0	0	100.0	1.69
28	Sweet potato ..	<i>Ipomoea batatas</i>	68.0	0.039	0	0	100.0	0.600
<i>Vegetables.—</i>								
29	Bitter gourd ..	<i>Momordica charantia</i>	91.5	0.015	0	0	100.0	0.450
30	Brinjal (red variety)	<i>Solanum melongena</i>	91.4	0.036	0	0	100.0	..
31	Cluster beans ..	<i>Cyamopsis psoralioides</i>	84.8	0.057	0	0	100.0	0.703
32	French beans ..	<i>Phaseolus vulgaris</i>	89.7	0.041	0	0	100.0	0.201
33	Lady's fingers ..	<i>Hibiscus esculentus</i>	84.3	0.080	0	0	100.0	..

34	Peas (green)	<i>Pisum sativum</i>	78.4	0.152	0.038	25	75.0	0.623
35	Ridge gourd	<i>Luffa acutangula</i>	95.7	0.037	0	0	100.0	2.7
36	Snake gourd	<i>Trichosanthes anguina</i>	97.1	0.029	0	0	100.0	2.0
<i>Leafy vegetables.</i> —										
37	Anarant	<i>Anarantus gangeticus</i>	85.5	0.024	0	0	100.0	0.530
38	Cabbage	<i>Brassica oleracea capitata</i>	93.5	0.024	0	0	100.0	0.710
39	Coriander	<i>Coriandrum sativum</i>	90.5	0.053	0	0	100.0	..
40	Fenugreek	<i>Trigonella fenugracum</i>	80.4	0.046	0	0	100.0	2.77
<i>Condiments, spices, etc.</i> —										
41	Fenugreek seeds	<i>Trigonella fenugracum</i>	10.2	0.520	0.127	24.0	76.0	0.050
42	Ginger	<i>Zingiber officinale</i>	80.0	0.062	0.0048	7.2	92.3	1.06
43	Tamarind (unripe)	<i>Tamarindus indicus</i>	70.0	0.025	0	0	100.0	..
	" (ripe)	" "	26.4	0.101	0	0	100.0	..
44	Poppy seeds	<i>Papaver somniferum</i>	3.6	0.874	0.313	35.8	64.2	0.270
<i>Fruits.</i> —										
45	Apple	<i>Pyrus malus</i>	85.1	0.013	0	0	100.0	..
46	Plantain	<i>Musa paradisiaca</i>	76.5	0.037	0	0	100.0	..

Note.—The analytical figures for cereals, pulses, oil seeds, and nuts are expressed on dry weight basis, while those for roots and tubers, vegetables, leafy vegetables, condiments, and fruits are expressed on a fresh weight basis.

DISCUSSION.

The data presented above show conclusively that a large part (50 to 70 per cent) of the phosphorus contained in cereals commonly used in India as food materials is present as phytin P, which is generally considered as not available for nutrition. Thus 50 to 70 per cent of P present in rice, ragi, and cholam is in the form of phytin. On polishing the cereals (rice and wheat) the total P, as well as the percentage of phytin P, decreases. Oil seeds and nuts also contain phytin in large amounts, while the percentage of phytin P contained in tubers is very little. Green vegetables and leafy vegetables do not contain phytin, thereby showing that the P contained in these foodstuffs is completely available for nutrition.

Further, the experimental results would show that the phytin content decreases on soaking the seeds in water. This is particularly of interest in view of the fact that soaking seeds, before they are used for the preparation of certain foods, is one of the items in culinary practices in India.

The phytin content of cereals is found to increase with the ripening of the grain, and the concentration of phytin seems to be highest with the lowest activity of the phytase present in the seed. Thus, it can be seen from the Table that the phytin content of immature maize is lower than that of mature maize of the same variety. Immature maize is therefore a better source of available P than the mature one. In view of the fact that in India maize is eaten by a large section of the poor, these observations are of interest in showing the superiority of the immature grain over that of the mature one, from the point of view of P availability.

The effect of cooking on the phytin content of ragi has been investigated and the results show that the change in the phytin content as a result of cooking is very little. Further work on the effect of cooking and other culinary practices on the phytin content of foodstuffs is in progress.

The unavailability of phytin P is an indication of the absence of enzymes in the intestine which hydrolyse phytin. Plimmer (1913) showed for the first time that phytin is not hydrolysed by the enzymes of the digestive tract of animals. Lowe and Steenbock (*loc. cit.*) also found no evidence for the enzymic hydrolysis of phytin by the extracts of the intestine from the rat and the chick. Recently, however, Patwardhan (1937) has reported that an enzyme capable of hydrolysing phytin is found in the intestines of albino rats, and that the extracts of the intestines of guinea-pigs and rabbits are inactive or very feebly active towards phytin. Even assuming that phytase is present in the intestines, the activity of the enzyme is very low compared to that of the enzyme present in fresh vegetables. The phytase of vegetables renders the phytin P available for nutrition by hydrolysing it into inorganic phosphorus. An examination of the results in the Table brings out some interesting correlations between the 'available' non-phytin P and phytase contents of foodstuffs. The phytase activities of tubers, roots, fresh vegetables, and leaves are very high compared to those of cereals which contain less than 50 per cent of the P as available non-phytin P. Thus, the higher the phytase activity the greater is the amount of non-phytin P content of foodstuffs. Foodstuffs which contain phytase, if consumed raw along with other cooked cereal foods, would bring about the conversion of phytin P, which is of poor nutritional availability into inorganic P which is an easily available form of P. The nutritionist might question the practical

significance of the presence of this enzyme in high concentrations in fresh vegetables, tubers, and leaves. The author believes that evidence presented in this paper, as well as several observations previously recorded in the literature (Lowe and Steenbock, *loc. cit.*), points to the great importance of this enzyme from the standpoint of phytin nutrition.

SUMMARY.

Cereals, pulses, oil seeds and nuts, vegetables and tubers, commonly used in Indian dietary, were tested for their total P, phytin P, and phytase content. A large part (50 to 70 per cent) of the phosphorus contained in cereals commonly used in India is present as phytin, which is not available for nutrition. Pulses, oil seeds, and nuts contain 20 to 60 per cent of the phosphorus as phytin P, while roots and tubers contain very little of phytin P. Green vegetables and leafy vegetables do not contain phytin, thereby showing that the phosphorus contained in these foodstuffs is completely available for nutrition.

The phytin content is found to increase on soaking the seeds in water. With the ripening of the grain there is an increase in the phytin content. Immature grains are therefore better sources of available phosphorus than mature ones. The phytase activities of tubers, vegetables, and leaves are found to be very high compared to those of cereals. The importance of this enzyme in phytin nutrition is discussed.

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Note added on 24th February, 1938.—Since the completion of this paper Sundararajan (1938) from the Nutrition Research Laboratories, Coonoor, has reported the results of his investigation on phytin-phosphorus content of Indian foodstuffs. There is a general agreement between the two sets of results on the phytin content of foodstuffs.—K. V. G.

- SUNDARARAJAN, A. R. (1938) .. *Ind. Jour. Med. Res.*, **25**, p. 685.

hand, egg-white is rich in the extrinsic factor, while B₆ is said to be absent (Miller and Rhoades, 1934). Yeast has been shown to be a potent source of all those factors but so far the absence of either B₆ or flavine in the diet of the rat has failed to produce any type of anæmia in this animal. The fact, however, that B₆ has been shown to be present in the muscle tissue of animals, birds, and fish would suggest that it is a component of human tissue and probably a dietary essential. The presence of B₆ in human muscle could of course easily be tested for by biological assay.

These facts, however, justify the analysis of Indian foods with a view to ascertaining by concomitant diet and clinical surveys in different areas whether there is a correlation between any morbid condition and the distribution of those substances in the diet. The data collected here include the flavine content of some 90 and the vitamin B₆ of some 40 Indian foodstuffs.

ESTIMATION OF FLAVINE.

Four different methods have been employed in the investigation of the flavine content of Indian foods, one biological assay, and three separate chemical processes. As regards the biological assay György (*loc. cit.*) has shown that on the rat the addition of 7γ flavine per day to an otherwise adequate diet is sufficient to cure the symptoms and produce a growth of 10 g. per week. This amount of flavine has then been taken to be present in that quantity of a foodstuff which permits a growth rate of 10 g. per week for four weeks. The following basal diet and supplements were given :—

	Per cent.
<i>Basal diet</i> —	
casein, B. D. H., light white	20
sucrose	69
coco-nut oil	3
McCollum's salt mixture.. ..	4
cod-liver oil	2
agar agar	2

Supplements—

B₁ extract equivalent to 2 g. yeast per day [prepared according to Kinnersley, O'Brien and Peters' (1933) method].

Wheat germ extract equivalent to 1 g. germ [prepared according to Birch and György (1936).]

The animals were kept on the diet until growth ceased and then the minimum quantity of the foodstuff which produced the standard rate of growth was ascertained.

Chemical methods.

Theoretically the amount of flavine present in an extract of a foodstuff could be estimated directly by the colorimeter were it not for the fact that many tissues

contain other colouring matter which vitiates the results. The elimination of those pigments can, however, be achieved in a large measure by adsorbing the flavine on Fuller's earth followed by elution. Van Eekelen and Emmerie (1935) found, however, that the complete elimination of other pigments was not attained. This was also our experience. There are known, however, to be great differences in the adsorption capacity of different adsorbents even for the same substance. For this reason it was considered advisable to adopt another technique. The estimation of flavine in the form of its irradiated product, lumiflavine, offers several advantages. Lumiflavine like flavine is both fluorescent and has a yellow colour. Lumiflavine is soluble in chloroform and hence can be separated from other pigments by solution in this medium and estimated colorimetrically or by the degree of fluorescence. A disadvantage attending this method lies in the fact that the complete transformation of flavine into lumiflavine is not attained—at least when small quantities are present. Possibly there is a certain amount of destruction of the flavine during irradiation. It is hoped, however, that the adoption of the technique worked out for the fluorescence measurement will eliminate this source of error.

A third chemical method consists in oxidizing an extract of tissue with acid potassium permanganate that destroys all pigments except flavine which can then be estimated directly in the colorimeter.

Technique of lumiflavine estimation.

The method employed is essentially that of Warburg and Christian as modified by Kuhn and Wagner-Jauregg (1935). One hundred grammes of the foodstuff were crushed and the juice expressed through muslin. Methanol was then added to the extract to bring the concentration up to 50 per cent. The pulp was then added, the whole acidified, boiled for ten minutes, and filtered. The pulp was again boiled in 50 per cent methanol and re-filtered. This process was then repeated until the filtrates were colourless. The combined extracts after washing in chloroform were then made N/2 alkaline with NaOH solution and placed under a 500-Watt lamp at a distance of 20 cm. to 30 cm. and exposed for two hours and twenty minutes. During irradiation the solution was surrounded by water and ice in order to keep the temperature below 20°C. The solution was then acidified to pH 4.0 with acetic acid and extracted repeatedly with chloroform until all the lumiflavine had been taken out. The chloroform solution after dehydrating with anhydrous sodium sulphate was concentrated at a low temperature over an electric light. During the process of extraction and concentration the lumiflavine was protected from light by means of black paper covering the flasks. This solution was then employed for the estimation of flavine both colorimetrically and by means of its fluorescence. The former was carried out with a Pulfrich photometer using filter 47 and E (lumiflavine) = $\frac{1 \text{ cm.}}{100 \text{ } \gamma/\text{c.c.}} = 4.3$.

For the estimation of the fluorescence of lumiflavine a special curve was made from irradiated flavine solutions and calibrated against a uranium glass plate using the Pulfrich photometer and an ultra-violet source of light. In the preparation of the graph pure crystalline lactoflavine was made up in seven different concentrations from 5 γ -10 γ —35 γ . These were all irradiated and extracted by

the method outlined above and after bringing to pH 4.0 calibrated against the uranium glass plate. The original concentration of lactoflavine taken was then plotted against the degree of fluorescence obtained from the lumiflavine. By this means it is hoped errors due to the partial transformation of flavine at different concentrations were eliminated.

Flavine estimation by means of oxidation with permanganate.

The method is essentially that of van Eekelen and Emmerie (*loc. cit.*). To 20 c.c. of the original 50 per cent methanol extract were added 2 c.c. glacial acetic acid and 2 c.c. saturated solution of potassium permanganate solution. After standing at room temperature for ten minutes 1 c.c. of H₂O₂ (6 per cent) was added, the solution filtered, and the flavine estimated colorimetrically in the photometer using filter 47 and $E(\text{flavine}) = \frac{5\gamma/\text{c.c.}}{2 \text{ cm.}} = 0.28$.

ESTIMATION OF VITAMIN B₆.

The biological assay of this factor is much more tedious and arbitrary than that of flavine. The animals must be kept on a diet absolutely free from B₆ and even then it takes some time before the symptoms develop. A number of different diets was tried until finally we bred young from specially fed mothers. Pregnant females were given the following diet: *khoi* (fried paddy), bread, milk, and carrots. This was continued until the tenth day of lactation when milk was given only twice a week. On weaning the young were put on the diet used in the flavine assay. 10γ crystalline B₁ and 10γ pure crystalline flavine were given daily as supplements. A discussion of the effect of different diets on the production of dermatitis will be treated in another paper. It will suffice to state that the above diet (coco-nut oil replacing hydrogenated vegetable oil) used by Bender and Supplee (1936) was found to be the most effective. An increase in the fat intake and a substitution of another carbohydrate for sucrose reduced the incidence of dermatitis. With this diet 75 per cent of the animals became affected in about five weeks. As soon as the typical symptoms appeared a small quantity of the foodstuff to be tested was given each day. If no improvement in the animal's general condition was noted in a day or two this quantity was immediately increased. It was not always possible to wait until the animal had developed a moderate or severe dermatitis as it often died without warning. In general, however, once we had tested a sample of each of the typical foods the amount of trial and error necessary to ascertain the minimum quantity to effect a cure was reduced. As the process of depletion was tedious and the rat could be used only once we were compelled to adopt this somewhat arbitrary technique. The unit of B₆ adopted by György (1935b) is contained in the minimum quantity of the foodstuff fed daily which is effective in curing the condition. The following method for expressing the number of units of B₆ has been adopted. The type of dermatitis was classified into three types, namely, mild, moderate, and severe. A unit of B₆ was said to be present in the minimum quantity of the food which effected a cure of the moderate type of dermatitis in seven days. Where the cure takes place in four, six, eight, and

nine days, etc., $+\frac{1}{4}$, $+\frac{2}{7}$, $-\frac{1}{4}$, $-\frac{2}{7}$ unit, etc., was added. Where the disease was mild or severe $+$ or $-$ 20 per cent was added to the above figure. The units of B₆ given in Table II have all been calculated on a 100 g. basis. It will be appreciated that this scheme is extremely arbitrary but it will suffice to show in general what foods are rich in this particular substance until such time as a suitable chemical technique has been worked out.

DISCUSSION.

The values obtained for flavine by all the different methods are given in Table I along with those found by other workers (Murthy, 1937) in Coonoor and in Germany (Kuhn and Wagner-Jauregg, *loc. cit.*). It will be noted that the average of the biological assay differs from those obtained by chemical means. This we think is due to the fact that the biological assay represents the mean of a considerable quantity of different samples of the foodstuff consumed over a period of time while the chemical estimation is carried out on one sample. In general we think that the average of the biological assay is the more reliable and for practical purposes the one to adopt in assessing a diet. There is always, however, the possibility to be kept in mind that the biological assay in view of its technique, i.e., rate of growth, may measure some other factor in addition. Among the foodstuffs which are rich in this factor are the pulses and legumes above all grams, and to a certain extent the leafy vegetables, namely, *pālang* and *puin saks*. The fruits and vegetables vary considerably but on the whole are distinctly poor in flavine.

As regards vitamin B₆ (Table II) from the few analyses available it is apparent that the cereals are a good source of this factor while fruits and vegetables, especially the orange and tomato, contain relatively little. Fish falls midway between.

During the progress of this work evidence has been accumulating (Edgar and Macrae, 1937; Halliday and Evans, 1937) which tends to confirm the presence in foodstuffs of a filtrate factor apart from flavine and B₆ which is necessary for the growth of the rat. The existence of this factor may possibly explain, to a certain extent, the higher values for flavine obtained by the assay as compared to the chemical methods. These animals received a wheat germ extract equivalent to 1 g. germ which undoubtedly contained this substance as evidenced by the response to flavine alone or in test foods but possibly in inadequate amount. Experiments are in progress to test the potency of the dose fed in those assays. Those findings, however, do not vitiate the results of the B₆ assay as the test depended on the cure of the specific condition, viz., dermatitis.

SUMMARY.

The estimation of the flavine content of 90 Indian foodstuffs has been carried out by biological assay and chemical methods. The estimation of vitamin B₆ in 28 Indian foodstuffs has been carried out by biological assay.

The pulses followed by the leafy vegetables are good sources while the fruits are poor in flavine. Much the same relative distribution has been noted for B₆, fish tissue, however, being a moderately good source.

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TABLE I.

No.	Name of foodstuff.	Botanical name.	BIOLOGICAL ASSAY (MILLI-GRAMME PER 100 G. OF FOOD-STUFF).		CHEMICAL METHODS (MILLIGRAMME PER 100 G. OF FOODSTUFF).				OTHER WORKERS' OBSERVATIONS (MILLI-GRAMME PTR 100 G. OF FOODSTUFF).	
			Range of values.	Average of all values.	Direct colorimetry.	Fluorescent method.	KMNO ₄ method.	Conoor Lab. chemical method.		German workers' chemical method.
	<i>Green leafy vegetables or saks.</i> —									
1	Pālang sāk (spinach)	<i>Spinacia oleracea</i>	0.155-0.196	0.1753	0.124	0.089	0.057	..
2	Puin sāk	<i>Basella rubra</i>	0.100-0.21	0.1570	0.159	0.143
3	Kólmi sāk	<i>Ipomoea reptans</i>	0.110-0.189	0.1492	0.116	..	0.086
4	Lettuce ..	<i>Lactuca sativa</i>	0.098-0.163	0.1310	0.128	0.116	0.326
5	Lāi sāk ..	<i>Amaranthus gangeticus</i>	0.035-0.063	0.055	0.034	0.045
6	Nótey sāk	"	0.030-0.056	0.049	0.025	0.045
7	Paltā sāk	<i>Trychosanthes dioica</i>	0.063-0.119	0.100
8	Lāo sāk (gourd leaves)	<i>Lagenaria vulgaris</i>	0.151-0.162	0.158	0.150
9	Kimrā sāk (pumpkin leaves).	<i>Cucurbita maxima</i>	0.080-0.150	0.140	0.138	0.068
10	Dhaniā sāk (coriander leaves).	<i>Coriandrum sativum</i>	0.059-0.120	0.098	0.0585
	<i>Vegetables.</i> —									
11	Potato ..	<i>Solanum tuberosum</i>	0.096-0.137	0.1093	0.048	0.043 (with peel).

TABLE I—*contd.*

No.	Name of foodstuff.	Botanical name.	BIOLOGICAL ASSAY (MILLI-GRAMME PER 100 G. OF FOOD-STUFF).		CHEMICAL METHODS (MILLIGRAMME PER 100 G. OF FOODSTUFF).			OTHER WORKERS' OBSERVATIONS (MILLI-GRAMME PER 100 G. OF FOODSTUFF).
			Range of values.	Average of all values.	Direct colorimetry.	Fluorescent method.	KMNO ₄ method.	
Vegetables—contd.								
12	Patól ..	<i>Trychosanthos dioica</i>	0.133-0.1498	0.1414	0.0536	0.060
13	Shinzā (ridge gourd)	<i>Luffa acutangula</i>	0.0357-0.103	0.0694	0.0132	0.012	0.022	..
14	Kurvā (pumpkin)	<i>Cucurbita maxima</i>	0.044-0.084	0.0473	0.023	..	0.022	..
15	Lāo (gourd)	<i>Lagenaria vulgaris</i>	0.0196-0.022	0.021	0.012	0.010	0.011	..
16	Carrot ..	<i>Daucus carota</i>	0.028-0.04	0.030	0.0177	0.02
17	Banana (green)	<i>Musa paradisiaca</i>	0.035-0.084	0.063	0.040	0.024	0.0028	..
18	Brinjal ..	<i>Solanum melongena</i>	0.057-0.063	0.060	0.057	0.040	0.051	..
19	Karelā (bitter gourd)	<i>Momordica charantia</i>	0.099-0.120	0.110	0.0900	0.087	0.016	..
20	Lady's finger	<i>Hibiscus esculentus</i>	Traces	..	Immeasurable	0.454
21	Tomato ..	<i>Lycopersicum esculentus</i>	0.035-0.049	0.048	0.036	0.041	0.0135	0.071
22	Cabbage ..	<i>Brassica oleracea</i>	0.050-0.067	0.0616	0.025	0.032	..	0.215
23	Cauliflower	<i>Brassica oleracea botrytes</i>	0.07-0.140	0.107	0.090	0.80	0.034	..
24	Ucheho ..	<i>Momordica charantia</i>	0.105-0.119	0.114	0.105	..	0.030	..
25	Cucumber	<i>Cucumis sativus</i>	Traces	..	0.0050	0.004

26	Slim (broad beans) ..	<i>Dolichos lablab</i>	0.068	0.068	0.059	0.050	0.025	0.560	..
27	Yens (green, fresh) ..	<i>Pisum sativum</i>	0.140	0.14	0.057	0.010	..	0.280	..
28	Radish ..	<i>Raphanus sativus</i>	Traces	..	0.020	0.020
29	Mann kóchu (arum) ..	<i>Colocasia antiquorum</i>	0.0210-0.010	0.035	0.029.5	0.022	0.020
30	Small kóchu (arum small variety).	"	Nil	..	0.030	0.015
31	Rāngā āloo (sweet potato).	<i>Ipomoea batatas</i>	0.019-0.056	0.053	0.07	0.044
32	French bean ..	<i>Phaseolus vulgaris</i>	0.021-0.049	0.032	0.043	0.030	0.014
33	Squash	0.060-0.075	0.07	0.071	0.065
34	Papaya (green) ..	<i>Carica papaya</i>	0.008-0.126	0.1211	0.1215	..	0.1215
35	Tunip ..	<i>Brassica rapa</i>	0.040-0.06	0.056	0.031	0.040	0.021
36	Ōh kōpi ..	<i>Brassica oleracea</i>	0.07-0.099	0.079	0.110	0.08
37	Beet ..	<i>Beta vulgaris</i>	0.115	0.115	0.0848	..	0.064
38	Figs (green) ..	<i>Ficus carica</i>	0.060-0.080	0.070	0.078	0.052
39	Jack-fruit (green) ..	<i>Artocarpus integrifolia</i>	Traces
<i>Cereals.</i> —									
40	Attā ..	<i>Triticum vulgare</i>	0.035-0.04	0.038	0.037	0.040	..	0.119	..
41	Cobs of corn (yellow)	<i>Zea mays</i>	0.063-0.07	0.0665	0.049	0.036	0.029
42	" (young cobs) ..	"	0.070-0.09	0.080	0.044	0.050	0.016
43	Rice (unpolished) ..	<i>Oryza sativa</i>	0.101	0.101	0.078	0.070	..	0.124	..
<i>Pulses and legumes.</i> —									
44	Lentil ..	<i>Lens esculenta</i>	0.28-0.35	0.32	0.068	..	0.044
45	Green mung ..	<i>Phaseolus radiatus</i>	0.105-0.110	0.107	0.0762	0.080

TABLE I—*contd.*

No.	Name of foodstuff.	Botanical name.	BIOLOGICAL ASSAY (MILLI-GRAMME PER 100 G. OF FOOD-STUFF).		CHEMICAL METHODS (MILLIGRAMME PER 100 G. OF FOODSTUFF).			OTHER WORKERS' OBSERVATIONS (MILLIGRAMME PER 100 G. OF FOODSTUFF).	
			Range of values.	Average of all values.	Direct colorimetry.	Fluorescent method.	KMNO ₄ method.		Conoor Lab. chemical method.
<i>Pulses and legumes—contd.</i>									
46	Gram (brown)	<i>Cicer arietinum</i>	0.200-0.466	0.3328	0.066	0.090	0.050	Traces	..
47	Arhar ..	<i>Cajanus indicus</i>	0.30-0.40	0.350	0.066	0.074	..	Nil	..
48	Black mung	<i>Phaseolus mungo</i>	0.301	0.301	0.059	0.0666
<i>Fruits.—</i>									
49	Grapes ..	<i>Citrus grandis</i> var. <i>maxima</i> .	0.021-0.025	0.023	0.080	0.024
50	Banana (chāmpā)	<i>Musa paradisica</i>	0.014-0.023	0.0187
51	" (Singapore)	"	0.049	0.049	0.034	0.048
52	" (kāntāli)	"	Traces	?	0.020	0.035	0.011
53	" (martamān)	"	0.010-0.038	0.014	0.062	0.040	0.02
54	Guava (Benares)	<i>Psidium guajava</i>	Traces-0.035	0.010	0.026	0.033
55	Pear ..	<i>Pyrus communis</i>	0.056-0.088	0.070	0.080	0.03	0.034
56	Papaya (ripe)	<i>Carica papaya</i>	0.014-0.021	0.0175	0.0169	0.0176	0.195
57	Orange ..	<i>Citrus aurantium</i>	0.040-0.06	0.050	0.0588	..	0.0409
58	Apple ..	<i>Pyrus malus</i>	0.021-0.039	0.028	0.040	0.030

59	Lemon juice	..	<i>Citrus medica</i> var. <i>limonum</i>	Nil	Nil	0.0014	0.005
60	Sank āloo	..	<i>Pachyrhizus angulatus</i>	Nil-Traces	Traces
61	Sugarcane (without skin).	..	<i>Saccharum officinarum</i>	0.050-0.069	0.063	0.0447	0.056	0.024
62	Plum (pear shaped)	..	<i>Prunus domestica</i>	0.049-0.076	0.070	0.044	..	0.019
63	" (circular)	..	"	0.030-0.045	0.010	0.025	..	0.060
64	Mango (green)	..	<i>Mangifera indica</i>	0.015-0.026	0.0175	0.030
65	" (ripe), Fazli	"	0.037-0.091	0.083	0.079	0.035	0.049
66	" langrā	..	"	0.012-0.050	0.046	0.080	0.030
67	" (Bombay)	..	"	0.035-0.04	0.0370	0.021
68	" Bengal	..	"	0.030-0.085	0.050	0.070	0.045
69	" totafuli	..	"	0.04-0.090	0.060	0.0184
70	Bael (ripe) (wood-apple)	..	<i>Feronia elephantum</i>	0.120 0.158	0.1344	0.168
71	Tamarind	..	<i>Tamarindus indica</i>	Traces	?
72	Jamrool	<i>Eugenia malaccensis</i>	0.0455	0.0455	0.0521	..	0.010
73	Gôlabjam	..	<i>Eugenia jambos</i>	0.098	0.098	0.0947
74	Melon	<i>Cucumis melo</i>	0.0313	0.0313	0.065	..	0.016
75	Water-melon	..	<i>Citrullus vulgaris</i>	0.0658	0.0658	0.020
76	Dates	<i>Phoenix dactylifera</i>	0.05-0.068	0.0644	0.059	0.030
77	Safedn	<i>Achras sapota</i>	0.020-0.059	0.028	0.060	0.0568	0.011
78	Apricot	<i>Prunus ameniaca</i>	0.196	0.196	0.104	0.088	0.057
79	Leechi	<i>Nephelium litchi</i>	0.0462	0.0462
80	Jām (black berries)	<i>Eugenia jambolana</i>	Traces-0.040	0.020	0.040	0.027	0.016

TABLE I—*concl'd.*

No.	Name of foodstuff.	Botanical name.	BIOLOGICAL ASSAY (MILLI-GRAMME PER 100 G. OF FOOD-STUFF).		CHEMICAL METHODS (MILLIGRAMME PER 100 G. OF FOODSTUFF).			OTHER WORKERS' OBSERVATIONS (MILLI-GRAMME PER 100 G. OF FOODSTUFF).
			Range of values.	Average of all values.	Direct colorimetry.	Fluorescent method.	KMNO ₄ method.	
<i>Fruits—contd.</i>								
81	Jack-fruit (ripe)	..	0.070-0.080	0.073	0.0645	0.034	0.044	..
82	Pine-apple	..	0.035-0.0425	0.038	0.013
83	Peach	0.063-0.07	0.066
84	Ata (custard apple)	Nil	Nil
85	Panifal (water chestnut)	..	Traces-0.06	0.066	Nil	..
86	Dāleem (pomegranate) juice.	..	0.070/100 c.c.	0.070/100 c.c.	0.08/100 c.c.	0.100/100 c.c.
87	Raisins	0.098	0.098
<i>Miscellaneous.—</i>								
88	Bazaar milk	..	0.063-0.099	0.087	0.080	0.090	..	0.10
89	Curd	0.087-0.099	0.088	0.082	0.060
90	Turmeric (powder)	..	0.14	0.140
91	Goat meat (dry)	..	0.070-0.084	0.077
92	Betel leaves	..	0.025-0.033	0.030	0.040	0.0308	..	1.26

TABLE II.

No.	Name of foodstuff.	Botanical name.	Units of B ₆ per 100 g.
<i>Green leafy vegetables or sāks.—</i>			
1	Lettuce	<i>Lactuca sativa</i>	25.6
2	Pālang sāk (spinach) ..	<i>Spinacia oleraria</i>	17.6
3	Paltā sāk	<i>Trychosanthes dioica</i>	27.4
4	Kumrā sāk (pumpkin leaves)	<i>Cucurbita maxima</i>	25.0
<i>Vegetables.—</i>			
5	Cabbage	<i>Brassica oleracea</i>	12.0
6	Tomato	<i>Lycopersicum esculentus</i>	14.0
7	Potato	<i>Solanum tuberosum</i>	25.0
8	Ucheche	<i>Momordica charantia</i>	17.4
9	Patōl	<i>Trichosanthes dioica</i>	20.6
10	Jhingā (ridge gourd) ..	<i>Luffa acutangula</i>	20.0
11	Kumrā (pumpkin) ..	<i>Cucurbita maxima</i>	9.5
<i>Cereals.—</i>			
12	Attā	<i>Triticum vulgare</i>	56.5
13	Rice (unpolished) ..	<i>Oryza sativa</i>	43.5
<i>Pulses and legumes.—</i>			
14	Gram	<i>Cicer arietinum</i>	152.0
15	Arhar	<i>Cajanus indicus</i>	50.0
16	Lentil	<i>Lens esculenta</i>	42.5
17	Black mung	<i>Phaseolus mungo</i>	56.5
<i>Fruits.—</i>			
18	Guava	<i>Psidium guajava</i>	27.4
19	Orange	<i>Citrus aurantium</i>	7.8 per 100 c.c.
20	Papaya	<i>Carica papaya</i>	22.6
21	Pear	<i>Pyrus communis</i>	23.0
22	Apple	<i>Pyrus malus</i>	19.0

TABLE II—*concl'd.*

No.	Name of foodstuff.			Botanical name.	Units of B ₆ per 100 g.
	<i>Animal foods.—</i>				
23	Hilsa fish	<i>Clupea ilisha</i>	24·3
24	Tangrā fish	<i>Mystus tengara</i>	50·6
25	Kātlā fish	50·0
26	Prawn	56·5
	<i>Miscellaneous.—</i>				
27	Yeast (dry)	506·0
28	Marmite	126·6

'AVAILABLE' IRON IN INDIAN FOODSTUFFS.

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THE recent growth of interest on the problem of nutrition has resulted in the assaying of various common Indian foodstuffs. But it may be pointed out that the usual method of ash analyses generally over-estimates the percentages of mineral present in them. This is especially true of iron since it has been shown by Elvehjem and his collaborators (1933, 1934*a* and *b*) that the hæmatopoietic value of a food is not represented by its total iron content but on the ionizable or 'available' portion of it (cf. Shackleton and McCance, 1936). The plant and animal tissues contain varying amounts of hæmatin iron which cannot be utilized for hæmoglobin formation (Elvehjem, 1932; Lintzel, 1928). These researches have further revealed that the available portion of iron in any food corresponds closely with the form of iron estimated by Hill's (1930) dipyriddy method. A knowledge of the iron content of some Indian foodstuffs would, therefore, be of importance not only for public health workers but also for clinicians as in treating cases of hypochromic anæmia, either available iron salts or foods rich in iron are now regarded as a specific remedy. This would further be of an additional interest as anæmia is present in a considerable percentage of Indian infants, growing children, pregnant women, and nursing mothers—the classes of the community which are particularly likely to suffer if their diet is not rich in 'available' iron.

Advantage was taken of the researches of Ranganathan *et al.* (1937) in selecting most of the materials to be analysed. The foodstuffs with which the experiments were carried out were all obtained from the local market; pâlâ was secured from the district of Barisal and soya beans from Darjeeling. In cases of pulses, rice, and similar products the materials were washed with distilled water and dried at about 37°C. The leafy vegetables were carefully washed and dried at room temperature in a dust-free atmosphere. The nuts, spices, and cereal foods were directly weighed for analysis. Thus, in short, each of the materials was treated in a way which is usually followed in its normal consumption.

EXPERIMENTAL.

Estimation of total iron.—The sample of the materials (about 2 g. to 5 g.) was weighed directly in a platinum crucible or basin and gently heated over a small flame. The temperature was gradually raised to red-heat when a dark mass was left behind. This was moistened with dilute sulphuric acid and heated slowly to obtain a white to light reddish-brown residue. It was once more treated with a drop of dilute sulphuric acid, heated slowly until fumes of sulphuric acid were evolved, and kept at that temperature for a few minutes. The solid thus obtained was dissolved in water and dilute iron-free ammonium hydroxide was added to bring the pH of the solution to near about 5. With the addition of hydroquinone (1 g.), the solution was filtered into a 100 c.c. measuring flask, 5 c.c. of a 0.2 per cent solution of α, α' dipyridine were added, and the volume was made up to the mark. After leaving aside for three to four hours the colour of the solution was compared with a standard made from ferrous ammonium sulphate (reagent quality), in a Klett colorimeter. In certain cases the results were verified by determining the iron percentages by the usual thiocyanate-amylalcohol extraction method.

Estimation of 'available' iron.—Here the method of Hill as modified by Köhler *et al.* (1936) was followed. The sample was cut into fine pieces in the case of soft materials or finely powdered in the case of dry materials in such a way that no contamination with any iron might occur. Two to five grammes in case of dried or hard materials and 10 g. to 25 g. in case of fresh and bulky products were taken in a stoppered measuring cylinder, and 10 c.c. of 10 per cent acetic acid, a gramme of chemically pure hydroquinone, and 5 c.c. of 0.2 per cent of α, α' dipyridine were added. The cylinder was then kept in an air-tight condition for 24 to 72 hours and then after the addition of 10 c.c. of 7.5 per cent lead acetate solution, it was again left over for 24 hours. It was subsequently either centrifuged or filtered and compared with the standard. In order to obtain an optically clear solution the addition of alcohol often seemed necessary before final filtration. The results obtained in both the estimations from an average of three samples are recorded in Table I. From the two sets the relative percentages of the 'available' iron in each of the food materials have again been calculated and recorded in the last column.

TABLE I.

NAME OF FOODSTUFF.			PERCENTAGE OF IRON, IN MG.		Relative percentages.
Bengali.	English.	Botanical.	Total.	'Available'.	
Chaul (siddha) ..	Rice, parboiled, home pounded.	<i>Oryza sativa</i>	1.05	0.88	83.8
"	Rice, parboiled, milled.	"	0.732	0.54	73.8
" (atap) ..	Rice, raw, milled	"	1.32	1.02	77.2

TABLE I—*contd.*

NAME OF FOOD-STUFF.			PERCENTAGE OF IRON, IN MG.		Relative percent-ages.
Bengali.	English.	Botanical.	Total.	'Avail-able'.	
Chira ..	Rice, beaten	<i>Oryza sativa</i>	5.5	1.46	33.4
Khoi ..	„ puffed	„	6.6	1.50	22.7
Moori ..	„ fried	„	5.5	1.65	30.0
Atta ..	Wheat	<i>Triticum vulgare</i>	5.5	2.74	49.98
Ruti ..	Brown bread	„	0.88	0.77	87.5
Sagu ..	Sago	<i>Metroxylon sago</i>	1.87	1.65	86.24
Pala (sati) ..	„	<i>Curcuma zoodoria</i>	5.12	3.7	72.3
Suji ..	Semolina	<i>Triticum vulgare</i>	2.96	1.77	60.0
Barley ..	Barley	<i>Hordeum vulgare</i>	2.50	1.25	50.0
Chhola ..	Horse gram	<i>Dolichos biflorus</i>	5.60	3.16	56.3
„ ..	Bengal gram	<i>Cicer arietinum</i>	9.4	5.84	62.12
Mung dhal (kacha)	Black gram (unroasted).	<i>Phaseolus mungo</i>	2.2	1.92	87.28
„ (roasted)	Black gram (roasted).	„	10.8	8.79	81.39
„ (sona) ..	Black gram (roasted).	„ var. Linn	7.33	5.78	78.85
Mas kabai ..	Green gram	<i>Phaseolus radiatus</i>	3.99	2.8	64.0
Arhar dhal ..	Red gram	<i>Cajanus indicus</i>	8.9	5.8	65.16
Moosur dhal ..	Lentil	<i>Lens esculenta</i>	3.14	2.64	84.09
„	Soya bean (black)	<i>Glycine hispida</i>	6.87	6.10	90.0
„	„ (white, small).	„	4.06	3.96	97.5
Motor suti ..	Peas (fresh)	<i>Pisum sativum</i>	1.34	1.15	85.82
„	French bean	<i>Phaseolus vulgaris</i>	1.08	0.798	73.89
Gajar ..	Carrot	<i>Daucus carota</i>	0.65	0.48	73.84
Ochhche ..	Bitter gourd	<i>Momordica charantia</i>	1.66	0.704	42.2
Dharos ..	Lady's fingers	<i>Hibiscus esculentus</i>	1.1	0.73	66.36

TABLE I—concl'd.

NAME OF FOODSTUFF.			PERCENTAGE OF IRON, IN MG.		Relative percent-ages.
Bengali.	English.	Botanical.	Total.	'Avail-able'.	
Kala (fresh) ..	Banana (unripe)	<i>Musa sapientum</i>	0.22	0.099	45.0
Palang sak ..	Spinach	<i>Spinacia oleracea</i>	6.4	2.42	37.8
Pudina ..	Mint	<i>Mentha viridis</i>	16.85	8.8	52.23
Puin sak	<i>Bassela cardifolia</i>	1.41	0.31	22.0*
Neem ..	Neem	<i>Azadirachta indica</i>	6.8	1.698	25.0
Chapa kala ..	Banana (ripe)	<i>Musa sapientum</i>	0.25	0.168	67.61
Martaman kala	"	"	0.23	0.176	76.5
Pesta ..	Pistachio nut	<i>Pistacia vera</i>	6.53	2.87	43.95
Badam ..	Almond	<i>Prunas amygdalis</i>	4.95	4.4	88.9
Akrote ..	Walnut	<i>Juglans regia</i>	3.03	2.2	72.6
China badam ..	Ground-nut (roasted).	<i>Arachis hypogea</i>	1.7	0.6	35.3
Jamrul	0.012	0.008	83.9
Pan ..	Betel leaves	<i>Piper betel</i>	2.6	0.97	37.3
Kakmachi	<i>Solanum nigrum</i>	8.8	3.87	43.97
Sarisa ..	Mustard seed	<i>Brassica juncea</i>	8.8	4.4	50.0
Tetul ..	Tamarind	<i>Tamarindus indicus</i>	3.8	3.1	81.58
Methi ..	Fenugreek seeds	<i>Trigonella fœnumgræcum</i>	15.488	7.04	45.45

DISCUSSION.

In the determination of 'available' iron by the above method a brownish colour often developed and, thereby, interfered in the final comparison of the coloured iron dipyrindine salt. In such cases readings were taken as soon as seemed reasonable. Another difficulty arose in removing the colouring principle present in certain materials, such as turmeric, dry pepper, dates, and black berry. The bright transparent colour characteristic of iron dipyrindine salt was not obtained even on using alcohol or trichloroacetic acid as precipitating agent. For this a study on the influence of other chemicals like sodium hydrosulphite, aluminium chloride, is being contemplated. It may be pointed out, however, that in certain common cases such as in the estimations from almond, bananas, beans, peas, and wheat the

results obtained in the present investigation approach very near to the observations made by Elvehjem and his collaborators.

The necessity of iron for the formation of hæmoglobin points to the importance of the question of the amount of this element essential for the daily human requirement. According to Sherman (1937) 12 mg. of food iron per day are considered to be a satisfactory standard. Of course in cases of normal adult males or non-pregnant females smaller amounts (*cf.* Farrer and Goldhamer, 1935) may be sufficient, but pregnant women, nursing mothers, or anæmic patients—where iron again is much better retained (Reimann and Schick, 1936; Whipple and Robschey-Robins, 1936)—may require more to maintain the average hæmoglobin level (*cf.* Coons and Coons, 1935). But as the availability of iron for physiological purposes varies widely in different foods, so it would be essential to know the amount of this element that would be afforded by an ill-balanced diet. On the basis of the present work the amount of iron that would be available for the organism from an ordinary diet as specified by Dr. Aykroyd in the Health Bulletin No. 23, issued by the Government of India, would be not more than 5.5 mg. (*vide* Table II) which would, naturally, not allow the margin of safety for possible poor retention or impaired utilization. The well-balanced diet of the said bulletin would, however, afford more than 13 mg. of 'available' iron (*vide* Table III). This diet consisting of the foodstuffs richest in iron together with certain other materials such as spices, betel leaves, etc., may supply the requisite amount of iron for maintaining an ideal blood picture under normal conditions. Other foods rich in iron are meat, meat products, and eggs, but they are costly and they are not usually favoured by Indians in general. It must be pointed out here that the iron content of foodstuffs would depend considerably upon the amount of soluble iron in the soil upon which the plants have been grown, and consequently, the amount available from any diet would depend not only on the type of foodstuffs but also on the locality and area from which they have been obtained.

TABLE II.

Name of foodstuff.	Amount, in oz.	'Available' iron content, in mg.	Total 'available' iron, in mg.
Rice (average) ..	15	3.45	5.27
Pulse (various) ..	1	1.36	
Non-leafy vegetables ..	1.5	0.281	
Green leafy vegetables ..	0.25	0.105	
Milk	1	0.072 (whole) (Ranganathan, 1937)	

TABLE III.

Name of foodstuff.	Amount, in oz.	'Available' iron content, in mg.	Total 'available' iron, in mg.
Rice (average) ..	10	2.300	13.28
Wheat, bread, sago, suji, etc.	5	2.550	
Pulse (various) ..	3	4.081	
Milk	8	0.576 (whole)	
Non-leafy vegetables (beans, carrot, gourd, green banana, lady's fingers, etc.).	6	1.124	
Green leafy vegetables (spinach, pui n s a k, neem, etc.).	4	1.676	
Fruits (bananas, almond, pistachio nut, walnut, etc.).	2	0.972	

Another factor which must also be considered is that this availability of iron in any foodstuff might increase on cooking or boiling the same in one or other container according to the usual custom before its consumption. Experiments are already in progress on this subject and it may be pointed out here that on boiling black gram (*Phaseolus mungo* var. Linn) in an iron pan its percentage of 'available' iron increased from 5.78 (*vide* Table I) to 12.3 mg. The fact that the percentage of iron in black gram (roasted) is always higher than that in the same pulse (unroasted) again points to the validity of the above statement. Consequently, a knowledge of the 'available' iron content of cereals, pulses, vegetables, etc., after cooking them in an earthenware, or in one of the more common metallic pans, seems to be of more importance from the nutritional standpoint.

Amongst the foodstuffs, barley, sago, semolina, and pâlâ (*Curcuma zeodoria*) which are often admixed with milk apparently to increase the food value of the infant dietary, the last-named substance is found to be richest in its 'available' iron content. Of course there is experimental evidence (Elvehjem, 1934a) to show that the mere addition of a cereal food to milk does not induce normal hæmoglobin formation. Still, however, for its higher percentage of iron the ingestion of pâlâ (*Curcuma zeodoria*) to children between the ages of one and two and a half years, when they are generally found to suffer from nutritional anæmia (Mackay, 1931), may be considered. In this connection it may be further pointed out that the percentages of calcium, phosphorus, and protein in these substances are 0.02, 0.015, and 0.46 respectively.

SUMMARY.

1. A number of cereals, pulses, and various other Indian foodstuffs has been investigated with reference to their contents of 'available' iron.

2. Though certain foods are rich in 'available' iron, the question rises whether an ordinary diet is sufficient for the supply of daily iron requirement, particularly for pregnant women, nursing mothers, and persons whose retention of iron is being hindered by any physiologic or pathologic condition.

3. In view of the fact that pâlâ (*Curcuma zedoaria*) is rich in iron it is suggested that the milk-cereal diets of children may be reinforced with this food.

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OBSERVATIONS ON THE BASAL METABOLISM OF INDIAN BOYS IN CALCUTTA.

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THE problem of the basal metabolic rate of races living in the tropics has been receiving a certain amount of attention for some time past. This interest has been due in part to a desire to know whether race or environmental conditions, especially temperature, can or does alter a biological process which in European races in a temperate climate has been shown to be remarkably constant for any particular age or sex. In Europe and the United States a mass of data has now been collected and standards of comparison laid down for adults and children living in a temperate climate. Further, the increasing interest which is now being paid to diet all over the world has directed the attention of workers to assessing the food requirements of individuals under different environmental conditions. One of the main factors determining the calorie requirements, it is generally accepted, is the basal metabolic rate. *A priori* it was expected that those people, indigenous or foreign, living in the tropics might show a lower basal metabolic rate (henceforth B. M. R.). Investigations hitherto carried out have by no means proved this conclusively nor shown definitely that there is a racial factor. Bose and De (1934) working in Calcutta with adults did not find that there was any very material difference. Krishnan and Vareed (1932) in Madras, however, found a reduction of the B. M. R. of 12 per cent and 15 per cent in men and women, respectively, as compared with the du Bois standards. Similar but smaller differences were recorded by Mason (1934) among women in Madras.

The problem of the B. M. R. of Indian children has not hitherto been investigated and the object of the observations recorded here is to stimulate work on this subject in different parts of India with a view to fixing standards for this country, especially in regard to caloric requirements. The subjects of this study were Hindu and Mohammedan boys of 6 to 16 years of age, and inhabitants of Calcutta. Their economic status was low, the pay of their fathers (clerical class) ranging from Rs. 30 to Rs. 70 per month. A comparison of their heights and weights with those of the school children of different classes examined previously by Wilson *et al.* (1937) shows them to be actually lighter and shorter than those school children of the lowest economic status observed by them.

METHOD.

The B. M. R. was determined by the Douglas bag method, the usual precautions as to the post-absorptive state being observed. The boys were called for at their house at about 6 a.m. and taken to the dwelling of the observer where after resting for about 30 to 40 minutes the B. M. R. was taken. In cases where the respiration increased markedly or signs of restlessness were noted during the experiment another or a third observation was made at some later date. The measurement and analysis of the expired air were carried out in an air-conditioned room (78°F.) of the Institute of Hygiene.

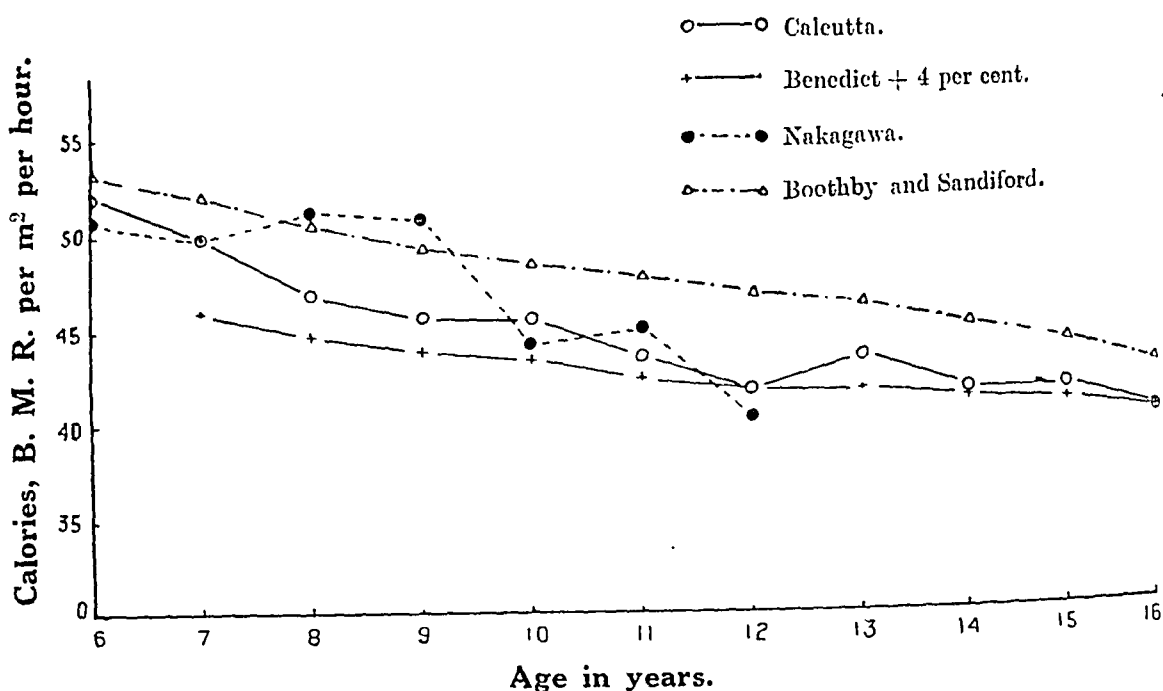
The data expressed in calories per square meter per hour and total calories per hour are shown in the Table and the Graph along with the corresponding standards of Aub and du Bois (1917), Benedict and Harris (1919, 1921), Boothby and Sandiford (1932) and Nakagawa for Japanese boys. The B. M. R. per hour has also been compared with Benedict's figures for boys of the same age (two ages only, 10 and 15) and the same height. A comparison has also been given between Benedict's figures for boys of the same weight, age being neglected. It will be seen that the figures obtained by us fall markedly below those of Aub and du Bois, — 9·7 per cent to — 19·5 per cent, and to a lesser degree below those of Boothby and Sandiford and Nakagawa. Compared with Benedict's figures, on the other hand, an increase is to be noted varying from + 10·9 per cent at the age of seven to less than 1 per cent as the age increases. Taken all over, above the age of eight our figures are never more than + 6 per cent above those of Benedict. Nakagawa's figures, although a little irregular, tend to approximate to ours. It should be noted that he was working on an Oriental race in contrast to the American observers. A comparison of our figures for the total metabolism per hour with Benedict's for boys of the same weight, age being neglected, shows a reasonable agreement except at lower ages. Considering the fact that the average weight of the Calcutta boys is much below that of the better classes of the same community (at the age of 12 they are some 10 lb. lighter) it is an open question whether they are not suffering from malnutrition. In this connection the observations of Blunt *et al.* (1921) on the metabolism of underweight children in the U. S. A. are of interest. They actually found the metabolism to be from 8 to 40 per cent above Benedict's original standard. This, of course, is not in keeping with the physiological effect of a deficient food intake or starvation (Lusk, 1928). There was no question, however, of the U. S. A. children being underfed as regards calories. Further, in the diet surveys in Calcutta households, although a defective diet was indicated, the caloric intake appeared

TABLE.

Average age.	Average body-weight.		Average height.		Basal metabolic rate (Calcutta observations) per m ² per hour.	Basal metabolic rate (Aub and du Bois) per m ² per hour.	Per cent variation.	Basal metabolic rate (Benedict + 4 per cent) per m ² per hour.	Per cent variation.	Basal metabolic rate (Boothby and Sandiford) per m ² per hour.	Basal metabolic rate (Nakagawa) per m ² per hour.	Total metabolism per hour. (Calcutta.)	Total metabolism per hour of boys of same weight (age neglected). (Benedict and Talbot.)	Respiratory quotient. (Calcutta observations.)
	Kg.	Lb.	Cm.	Inches.										
6	16.4	36.08	109.6	44.7	51.85	53.0	50.82	36.22	31.5	0.79
7	16.55	36.41	110.8	45.2	50.16	58.0	-9.7	46.0	+10.9	52.0	50.38	35.81	32.0	0.79
8	19.09	41.99	117.6	48.0	47.28	57.0	-18.3	45.0	+5.0	51.0	51.62	37.92	34.6	0.82
9	21.31	46.88	124.7	50.8	45.59	55.0	-16.6	44.8	+1.0	50.0	52.09	39.74	37.0	0.82
10	22.19	48.81	127.1	51.8	46.06	52.5	-11.3	44.0	+2.0	49.0	44.83	41.37	37.9	0.83
11	25.41	55.90	131.4	53.6	44.16	52.0	-15.2	43.5	+1.0	48.5	45.01	42.75	41.7	0.84
12	28.58	62.87	139.2	56.8	42.81	52.5	-19.5	42.5	+0.8	47.5	41.02	45.72	45.1	0.81
13	28.63	62.98	138.2	56.4	44.92	53.0	-15.3	42.0	+6.1	47.0	..	48.36	45.2	0.80
14	33.36	73.39	146.5	59.7	42.33	51.0	-17.0	42.0	+0.7	46.0	..	50.47	49.4	0.83
15	41.18	90.59	159.2	64.9	42.31	47.0	-10.0	41.0	+3.1	45.0	..	58.06	..	0.88
16	41.02	90.24	161.6	65.9	41.10	46.0	-10.7	40.5	+1.4	44.0	..	56.82	..	0.81

GRAPH.

Comparing B. M. R. of Calcutta boys with other standards.



to be reasonably adequate. Possibly some such factor as was observed by Blunt *et al.* (*loc. cit.*) is playing a part here and obscuring a real reduction in B. M. R. It should be pointed out that observations in Calcutta (Bose and ourselves) tend to show that there is not a great or any decrease in the B. M. R., while corresponding observations in Madras on adults (Mason, *loc. cit.*; Krishnan and Vareed, *loc. cit.*) do show this feature. In this connection it should be pointed out that the average physique in the Madras Presidency appears to be poorer than in Calcutta. The average weights of our children, the lowest noted in Calcutta, are approximately those noted by Aykroyd and Krishnan (1937) in a group of boys in a relatively affluent class in rural Madras (Mayanur district). Aykroyd also observed in his surveys caloric intakes much below what we have observed here and indeed some appeared to be taking a quantitatively insufficient diet. Possibly some such factors as these may be concerned in the discrepancies observed. Further work is urgently required from other parts of India and on children who are known to be getting a reasonably adequate diet or one which approaches qualitatively to European standards. The data shown here may be said to indicate either a reduction or an increase of metabolism according to the standards employed. With one exception they fall below the standards obtained in the U. S. A. on boys of European origin. On the other hand, they fall within the two extremes quoted, namely those of du Bois and Benedict. We are inclined to think that they may be on the low side, but taking into consideration their physique and diet there is no evidence that a racial factor is involved.

SUMMARY.

The basal metabolic rate of 62 (mostly Hindu) boys of ages 6 to 16 from a poor class in Calcutta has been studied. The results are lower in varying degrees when compared to the figures of Aub and du Bois, Boothby and Sandiford, and Nakagawa, while somewhat higher than those of Benedict (modified).

Taking all standards into consideration the figures might justify the conclusion that the metabolism tends to be on the low side. Considering the fact that the boys are of a poor class, underweight and on a low-protein diet, it would appear unjustifiable to favour the hypothesis of a racial factor being the cause.

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PROTOCOLS OF EXPERIMENTAL DATA.

Age.		Weight (ag.).	Height (cm.).	Pulse.	Respira- tion.	CO ₂ consumption per minute (c.c.).	O ₂ consumption per minute (c.c.).	R. Q.	Total B. M. R. per hour (cal.).	B. M. R. per hour per m ² (cal.).
Years.	Months.									
6	0	14.09	102.20	94	30	85.20	113.0	0.75	32.74	52.21
6	0	17.28	120.30	100	43	102.50	133.6	0.77	38.86	50.79
6	0	16.14	109.20	83	28	99.18	124.5	0.80	36.44	52.06
5	7	17.50	108.20	100	36	106.40	130.2	0.82	38.30	53.06
6	3	15.68	107.95	86	33	99.63	118.7	0.81	34.78	51.15
AVERAGES ..		16.14 (35.51 lb.).	109.6 (43.15 ins.).	93	34	98.58	124.0	0.79	36.22	51.85
7	5	16.82	114.3	100	30	99.83	134.5	0.74	38.90	52.48
7	3	20.00	116.5	80	23	107.80	124.7	0.86	36.89	46.12
7	0	15.00	105.7	96	30	93.85	116.3	0.81	34.08	51.25
7	0	17.04	109.9	96	33	100.20	122.2	0.82	35.88	49.70
6	7	13.87	106.0	104	27	86.91	114.5	0.76	33.30	51.23
AVERAGES ..		16.55 (36.41 lb.).	110.8 (43.62 ins.).	95	29	97.72	122.4	0.79	35.81	50.16

8	4	20-46	122-6	86	40	105-50	122-8	0-86	34-96	40-83
8	3	20-00	120-0	84	21	109-30	145-1	0-75	42-05	51-28
8	0	19-32	118-0	88	35	104-40	135-9	0-77	39-52	49-78
8	0	19-32	120-3	90	26	100-96	128-4	0-79	37-49	46-28
7	8	18-41	112-7	88	35	105-20	126-5	0-83	37-21	47-21
8	0	19-03	114-3	94	25	114-20	130-3	0-88	38-68	48-35
8	0	17-05	115-6	92	23	105-90	120-6	0-88	35-80	47-73
AVERAGES ..		19-09 (42 lb.).	117-7 (46-3 ins.).	89	29	106-50	129-9	0-82	37-92	47-28
9	5	20-91	123-5	82	28	113-5	132-6	0-87	39-30	45-69
9	0	22-73	123-8	86	20	114-2	145-5	0-79	42-48	47-21
9	1	20-63	120-0	90	25	115-2	138-0	0-83	40-59	48-32
9	4	20-91	128-0	106	28	106-2	122-7	0-87	36-36	41-32
9	2	21-36	128-0	92	22	105-5	137-5	0-77	39-99	45-44
AVERAGES ..		21-31 (46-88 lb.).	124-7 (49-09 ins.).	91	25	110-9	135-3	0-82	39-72	45-59

PROTOCOLS OF EXPERIMENTAL DATA—*contd.*

Years.	Age. Months.	Weight (ag.).	Height (cm.).	Pulse.	Respira- tion.	CO ₂ consumption per minute (c.c.).	O ₂ consumption per minute (c.c.).	R. Q.	Total B. M. R. per hour (cal.).	B. M. R. per hour per m ² (cal.).
10	3	23.63	132.0	80	26	129.6	160.0	0.81	46.89	49.37
10	0	20.00	118.4	81	20	105.7	119.0	0.88	35.50	43.30
10	4	23.86	128.9	82	26	136.7	153.8	0.89	45.75	48.15
9	8	19.77	125.1	70	22	107.7	137.9	0.78	40.19	47.30
10	5	25.23	130.8	81	24	120.2	164.5	0.79	48.03	49.51
10	2	20.91	128.2	78	18	110.7	130.3	0.85	38.48	43.72
10	0	22.10	129.0	80	32	107.4	123.5	0.87	36.13	41.08
AVERAGES ..		22.19 (48.82 lb.).	127.2 (50.07 ins.).	79	24	118.1	141.3	0.83	41.57	46.06
11	2	29.50	137.2	60	33	141.2	173.9	0.81	50.97	47.20
11	0	25.91	125.7	79	26	132.1	154.3	0.86	45.64	48.56
11	0	22.96	129.2	82	24	115.4	136.3	0.85	40.25	44.24
11	2	25.91	138.1	64	20	101.8	121.4	0.81	35.78	35.08
10	6	22.73	127.0	74	24	121.2	138.8	0.87	41.13	45.70
AVERAGES ..		25.40 (55.88 lb.).	131.4 (51.73 ins.).	72	25	122.3	144.9	0.84	42.75	44.16

11	6	28.64	139.7	65	30	134.7	171.5	0.79	50.07	46.36
11	11	25.00	132.0	76	20	108.8	138.2	0.79	40.33	41.59
11	6	30.91	142.9	74	24	137.6	165.4	0.89	46.22	41.27
11	11	28.64	140.1	68	24	127.2	143.6	0.89	42.72	39.56
12	3	30.23	142.2	62	22	118.1	147.6	0.80	43.16	38.90
11	7	32.28	138.5	72	19	133.3	168.1	0.79	49.08	43.82
12	2	23.86	138.8	70	19	127.7	148.7	0.86	43.98	44.88
12	2	29.09	139.7	75	19	127.7	173.7	0.74	50.23	46.09
AVERAGES ..		28.58 (62.88 lb.).	139.2 (54.8 ins.).	70	22	126.9	155.9	0.81	43.72	42.81
12	6	37.27	151.1	74	24	165.1	199.2	0.83	58.60	45.78
13	5	25.45	135.2	68	23	132.7	163.5	0.81	47.93	46.09
13	0	29.54	139.4	90	22	134.6	171.5	0.78	49.98	45.85
13	4	21.13	126.4	70	20	104.7	125.8	0.83	37.00	42.05
12	6	29.77	138.8	72	22	130.9	165.8	0.79	48.41	44.05
AVERAGES ..		28.63 (62.99 lb.).	138.2 (54.4 ins.).	75	22	135.6	165.2	0.80	48.38	44.92

PROTOCOLS OF EXPERIMENTAL DATA—*concl'd.*

AGE.		Weight (ag.).	Height (cm.).	Pulse.	Respira- tion.	CO ₂ consumption per minute (c.c.).	O ₂ consumption per minute (c.c.).	R. Q.	Total B. M. R. per hour (cal.).	B. M. R. per hour per m ² (cal.).
Years.	Months.									
14	0	40.90	157.5	58	29	141.6	180.6	0.78	52.62	38.69
14	3	35.00	151.1	64	24	159.4	181.8	0.88	53.98	43.88
13	6	39.09	152.1	86	15	176.9	207.7	0.85	47.83	41.96
14	3	32.05	147.3	72	19	131.5	163.2	0.81	47.83	41.96
14	0	19.77	134.6	85	24	105.3	123.9	0.85	36.59	40.66
AVERAGES ..		33.96 (73.79 lb.).	146.5 (57.6 ins.).	73	22	142.9	171.4	0.83	50.47	42.33
15	3	41.81	155.9	81	23	175.1	214.2	0.82	62.89	46.24
15	0	36.93	156.3	72	24	174.4	191.7	0.91	57.06	43.89
15	4	45.45	169.2	68	23	177.1	200.6	0.88	59.59	39.97
15	0	42.28	152.4	70	21	176.2	185.3	0.95	55.73	41.28
15	2	39.43	162.5	74	20	158.9	186.4	0.85	55.04	40.18
AVERAGES ..		41.18 (90.59 lb.).	159.2 (62.68 ins.).	73	22	172.3	195.6	0.88	58.06	42.31
16	5	48.64	158.8	60	26	162.5	207.5	0.78	60.47	40.76
16	0	44.10	169.2	81	24	160.4	192.8	0.83	56.71	38.32
15	8	27.73	146.7	86	24	137.9	158.9	0.87	47.09	43.20
15	6	40.68	166.1	70	23	155.2	201.3	0.77	58.65	41.60
16	3	43.86	167.0	78	18	171.9	208.1	0.83	61.21	41.63
AVERAGES ..		41.02 (90.24 lb.).	161.6 (63.62 ins.).	75	23	157.6	193.7	0.81	56.82	41.10

ENZYME METHOD FOR THE ESTIMATION OF ADRENALINE IN SUPRARENAL GLANDS.

BY

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Numerous chemical and biological methods are available in literature for the estimation of adrenaline, the active principle of the suprarenal medulla. Most of the chemical methods are based on the formation of coloured compounds, when adrenaline is made to react with certain substances. Thus, the ease with which adrenaline is oxidized by oxidizing agents, viz., iodine, iodic acid, mercuric chloride, persulphates, brown oxides of manganese, etc., to a red coloured compound has given rise to numerous methods for its assay. The green coloration with ferric chloride due to the presence of catechol nucleus in adrenaline has also been employed for its determination. The method which is frequently employed is due to Folin, Cannon and Dennis (1913) in which the colour reaction depends upon the reduction of phosphotungstic acid to lower, deep blue oxides of tungsten. The method is quite delicate but was found by Lewis (1916) and several other workers to give results which did not agree with those obtained by the biological method. Experiments in the present work have also confirmed this observation. Much more adrenaline was found in the suprarenals than the biological method revealed. According to Rees (1936) the higher values are due to ascorbic acid, but nothing definite is yet known regarding the nature of the reducing substances other than adrenaline present in the suprarenals.

It has been found by Neuberg (1908) that an enzyme from the ink-bag of *Sepia officinalis* produces a black pigment from adrenaline, and Abderhalden and Guggenheim (1908) observed that adrenaline solutions are coloured red by a tyrosinase from the fungus *Russula delica*. It has been also observed by me that an oxidase from the seeds of *Dolichos lablab* has the property of oxidizing adrenaline to a red-coloured compound and, based on this observation, a colorimetric method has been developed for its assay. Recently Blaschko, Richter and Schlossmann (1936) detected the presence of adrenaline oxidase in the liver and kidney of rats, rabbits, and guinea-pigs which was found to oxidize adrenaline molecules in the side chain (Blaschko *et al.*, 1937). Further Green and Richter (1937) found that adrenaline could be rapidly oxidized by cytochrome and by the indophenol-cytochrome system. They have isolated the oxidation product adrenochrome in a crystalline condition.

The present investigation was undertaken with a view to standardize the conditions for the colorimetric determination of adrenaline by the enzyme method.

EXPERIMENTAL.

The solutions required for the experiment were prepared as follows :—

Standard adrenaline solution.—It was prepared by diluting 5 c.c. of adrenaline chloride solution (1 : 1,000, Parke, Davis and Co.) to 100 c.c. The solution was preserved in the cold.

Enzyme solution.—One hundred grammes of meal from *Dolichos lablab* which was found to be a rich source of the oxidase were extracted with 1,000 c.c. of 5 per cent saline at 0°C. for 24 hours. The solution was filtered and dialysed in collodion bags against distilled water till free from the salt. The dialysate was filtered, the filtrate preserved under toluene in the cold, and used as a source of the enzyme.

The reaction mixture consisted of 5 c.c. of the standard adrenaline solution and 2 c.c. of the enzyme solution. It was observed that by the addition of a few drops of dilute hydrogen peroxide the reaction could be hastened as judged by the appearance of red colour; so in all the subsequent experiments three drops of 1 per cent hydrogen peroxide were added.

With a view to study the dependence of the reaction on pH of the reaction mixture, 5 c.c. of the standard adrenaline solution were mixed with buffers of varying pH. It was observed that the colour formation took place between pH 4.4 and pH 7.6 and the colour was more stable in the acid range. Further, at pH 6.0, the colour in the presence of hydrogen peroxide appeared within one minute and seemed to remain sufficiently stable for a colorimetric comparison. Hence, in the subsequent experiments, the pH of the reaction mixture was adjusted to 6.0 by the addition of M/2 Sorensen's phosphate buffer of pH 6.0.

The kinetics of this reaction was then studied at pH 6.0 with standard adrenaline solutions and the extract of fresh suprarenal glands from dogs. Five c.c. of the solution were mixed with 2 c.c. M/2 phosphate buffer of pH 6.0, three drops of 1 per cent hydrogen peroxide, and 2 c.c. of the enzyme. The colour developed was compared with an artificial standard containing the following dye-stuffs in the given portions :—

3 c.c. of 0.1 per cent Bismark brown	} Volume made up to 100 c.c.
0.1 c.c. of 0.1 per cent neutral red	

Table I incorporates the data.

Thus, it will be seen that the colour development is complete within four minutes and it remains stable for 15 to 20 minutes.

In order to see the validity of the method, different concentrations of adrenaline were employed, keeping (a) the volume of the enzyme solution and (b) the total volume of the reaction mixture the same. It was found that a good proportion exists between different concentrations of adrenaline and the intensities of the colour developed and the method can be employed to estimate adrenaline in concentrations up to 1 : 500,000.

TABLE I.

Time in minutes.	COLORIMETER READINGS IN MM. WHEN THE ARTIFICIAL STANDARD WAS SET AT 20.		Time in minutes.	COLORIMETER READINGS IN MM. WHEN THE ARTIFICIAL STANDARD WAS SET AT 20.	
	Adrenaline solution (P. D. & Co.).	Suprarenal extract.		Adrenaline solution (P. D. & Co.).	Suprarenal extract.
1½	18.5	..	10	13.7	14.3
2	16.5	19.7	15	13.7	14.3
3	13.7	15.1	20	13.7	14.5
4	13.7	14.3	25	14.0	..
5	[13.7	14.3	30	14.5	14.8
6	13.7	14.3	40	15.2	..

ASSAY OF ADRENALINE FROM FRESH SUPRARENAL GLANDS.

The fresh glands from dogs were ground with sand and extracted with N/10 HCl (6 c.c. per 0.5 g. gland) and the extract was brought to boil. Two c.c. of 10 per cent sodium acetate were then added, again brought to boil, cooled, and filtered. The filtrate was made to a known volume (25 c.c.), aliquots of which were employed for the estimation of adrenaline by (i) enzyme, (ii) chemical, and (iii) biological methods.

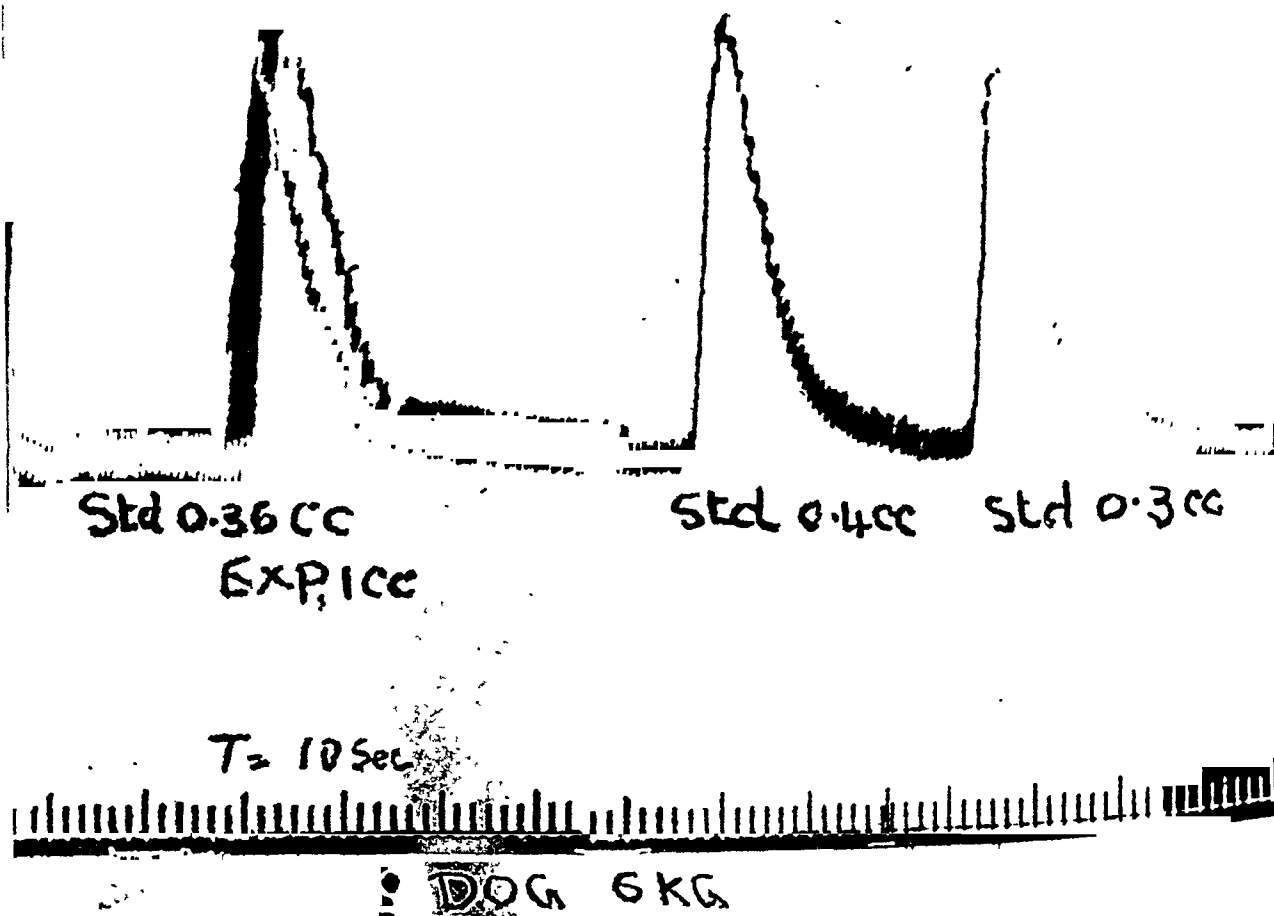
(i) *Enzyme method*.—Five c.c. of the solution were mixed with 2 c.c. M/2 Sorensen's phosphate buffer (pH 6.0), three drops of 1 per cent hydrogen peroxide, and 2 c.c. of the enzyme solutions. The colour developed was compared after five minutes with the standard prepared in the same way.

(ii) *Chemical method*.—A suitable aliquot of the solution was employed for the estimation of adrenaline by the well-known colorimetric method of Folin, Cannon and Dennis (*loc. cit.*).

(iii) *Biological method*.—The biological assays were made on dogs and rabbits by the blood-pressure method. Some results obtained by experiments on dogs and rabbits were repeated in cats by following Elliot's (1912) method and it was found that the results were identical. In all animals the brain was destroyed, the animal kept on artificial respiration, and sufficient atropine administered to paralyse the cardiac vagus endings. The blood pressure maintained a constant level within

about half an hour. The vasomotor response was checked by injecting known quantities of standard adrenaline solutions and noting that equal quantities produced the same degree of rise in blood pressure. At least 5 minutes' interval was allowed between two successive injections. A sufficient quantity of the suprarenal extract to be assayed was then injected intravenously so as to give a good but submaximal response. Different quantities of standard adrenaline solution were then injected in the same way and the amount required to produce the same rise in blood pressure as that produced by the suprarenal extract was determined. The amount of adrenaline present in the gland was then obtained by necessary calculations. A typical record is illustrated in the Figure:—

FIGURE.



♀ Dog—6 kg. Brain and spinal cord destroyed. Artificial respiration. Tracings from above downwards. Carotid blood pressure, time 10 seconds' interval and base line. Note that 1 c.c. of suprarenal extract given intravenously and 0.36 c.c. of a standard (1 c.c. = 0.05 mg.) adrenaline solution produce an equal rise in blood pressure. Note also that 0.4 c.c. of standard adrenaline produces a slightly higher rise and 0.3 c.c. a slightly lower rise in blood pressure than that produced by 1 c.c. of the extract.

The results obtained by these methods are presented in Table II:—

TABLE II.

Mg. of adrenaline per g. weight of fresh gland from dog.

Chemical method.	Biological method.	Enzyme method.	Chemical method.	Biological method.	Enzyme method.
0.64	0.48	0.49	1.33	0.64	0.69
0.97	0.53	0.51	1.07	0.83	0.75
0.65	0.58	0.89	2.01	2.10	1.88
1.15	1.25	0.93	0.98	0.26	..
0.65	0.34	0.33	1.14	0.88	0.82
1.13	0.54	0.44	2.00	1.54	1.39
0.82	0.50	0.50	1.75	1.31	1.28
1.29	0.61	0.45	1.50	0.85	0.74
1.15	0.70	1.04	1.53	0.87	0.91
0.51	..	0.51	2.60	2.30	2.22
1.83	1.47	1.05	..	0.39	0.37
1.87	1.17	1.05	1.40	1.60	1.40
1.54	0.83	0.81			

It will be seen from the above table that there exists a good agreement between the biological and enzyme methods, but by the chemical method large variations are obtained. These variations might perhaps be due to ascorbic acid (Rees, *loc. cit.*) or some other reducing substances whose nature is not yet known. The adrenaline content of suprarenal glands will naturally vary from animal to animal. The large degree of variation seen in my experiments may, however, be due to the fact that the animals from which these glands were removed were not under identical physiological conditions. Many of them were under a general anæsthetic and the nature of the anæsthesia as well as its duration varied. Some glands were removed from the animals after the termination of a physiological experiment. As the object of the experiments was a comparison of the new method with the biological method and not the adrenaline content of the suprarenals of dogs, these variations were found to be helpful.

Attempts are now being made to extend the method for the estimation of adrenaline from suprarenal glands of different species of animals.

SUMMARY.

An enzyme method for the estimation of adrenaline colorimetrically has been described. The method has been based on the oxidation of adrenaline by the enzyme to a red-coloured compound.

It has been shown that (i) the colour formation takes place between pH 4.4 and pH 7.6 and the colour is more stable in the acid range, (ii) the colour develops within one minute, (iii) good proportionality exists between different concentrations of adrenaline, and (iv) the method is sensitive to 1 : 500,000 concentration of adrenaline.

The method has been employed for the estimation of adrenaline from dogs' fresh suprarenal glands. A good agreement was found to exist between the biological and enzyme methods. The chemical method gave variable results.

ACKNOWLEDGMENTS.

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THE RÔLE OF FORMALIN IN THE ESTIMATION OF NITROGEN IN BODY FLUIDS.

BY

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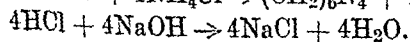
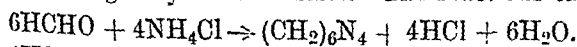
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IN the standard method of estimation of nitrogen in the body fluids, the nitrogen is converted into ammonium salts and the ammonia may be either liberated by alkali, absorbed in excess of standard acid and estimated by back-titration or the ammonia may be estimated colorimetrically by Nesslerization. In our laboratory the back-titration method is followed for urea and the colorimetric one for N. P. N., albumin, etc.

But in both these standard processes we come across certain difficulties to be discussed in detail later on. Briefly, in the back-titration method the results sometimes become obviously doubtful, and, in absence of some method of controlling the results, we have to repeat the whole experiment to get the correct ones. In the colorimetric method again, it is found that, in addition to its other minor disadvantages, the process of Nesslerization does not work very smoothly at times. For these reasons it was thought necessary to find out some method which would control the doubtful results of back-titration and replace the process of Nesslerization. The idea of using formalin in this connection came to our mind.

It is well known that, when a neutral solution of formalin is added to a neutral solution of ammonium salts, an equivalent amount of acid is liberated. If this acid is estimated by a standard alkali, the amount of alkali required gives the amount of ammonia originally fixed as salts. The reactions take place as follows :—



These reactions have been applied by Malfatti (1908) in estimating ammonia-nitrogen in urine. But since then it does not seem to have been applied in any other case.

The actual procedure with formalin is as follows:—

The solution of ammonium salts, obtained after digestion of the material, is diluted and neutralized. About 5 c.c. of formalin is also neutralized. The two are mixed together and the whole stirred well for a few minutes. The acid liberated is then titrated with a standard alkali and from the amount of this alkali required the nitrogen is calculated. The comparative results obtained by the standard method of back-titration and the formalin method have been given in Table I. The digestion mixture used for this purpose contains 50 c.c. of 5 per cent copper sulphate solution, 450 c.c. of pure concentrated sulphuric acid, and 500 c.c. of distilled water. Phosphoric acid is not used in it, because the mixture of phosphoric and sulphuric acids acts more rapidly on glass than concentrated sulphuric acid alone, and also that the presence of phosphates has been found to interfere with the exact end-point.

As mentioned before, this method has been applied either for controlling the doubtful results of back-titration or for replacing the method of Nesslerization. In both these cases, it has given successful results (*vide* Tables II and III).

Furthermore, to test the accuracy of this method, control estimations with formalin were carried out with very accurately measured quantities of pure urea and the results obtained as shown in Table IV were highly satisfactory.

DISCUSSION.

In the process of back-titration the chief disadvantage which we experience is that occasionally the results, for some reason or other, become either too high or too low and we have to face immense practical difficulties to get the correct results. But since the adoption of formalin for controlling these results such cases have become rare. The idea of using formalin here is that the ammonium salts present in the solution remain intact after final titration by the standard method and formalin is now made to react with them.

In the process of Nesslerization the disadvantages are numerous. It is expensive, elaborate, takes a good deal of time and, above all, it does not work very satisfactorily at times. Either a clumsy precipitate or a wrong colour often disturbs the experiment during colorimetry, whereas if formalin is used there are no such difficulties at all. Moreover, the process is quick, economical, equally accurate, and always works smoothly.

Table I shows the accuracy of the results obtained with formalin as compared with those of the standard back-titration method. In this case the same sample of blood was used for both the experiments.

In Table II we have shown the use of formalin in controlling the doubtful results of back-titration. In all the cases the results of formalin method were corroborated by repeating the standard experiment with the same sample of blood (col. 4).

Table III shows its value as a substitute for the process of Nesslerization.

In all the Tables it will be found that the results by the formalin method follow very closely the corresponding results obtained by the standard methods.

Table IV shows the results obtained by formalin method with known quantities of urea.

TABLE I.

Comparative results obtained by standard method of back-titration and the formalin method.

Case number.	Urea in mg. per 100 c.c. by back-titration method.	Urea in mg. per 100 c.c. by formalin method.	Case number.	Urea in mg. per 100 c.c. by back-titration method.	Urea in mg. per 100 c.c. by formalin method.
1	40	41	16	42	42
2	26	28	17	45	46
3	30	28	18	56	54
4	36	36	19	143	145
5	54	54	20	25	26
6	224	226	21	21	20
7	32	33	22	22	23
8	42	43	23	30	31
9	260	256	24	56	57
10	31	32	25	20	21
11	26	27	26	40	39
12	25	23	27	26	25
13	50	52	28	19	19.5
14	32	30	29	62	63
15	38	37	30	25	25

TABLE II.

Showing use of formalin in controlling the doubtful results of back-titration.

Case number.	Urea in mg. per 100 c.c. by back-titration.	Urea in mg. per 100 c.c. by formalin method.	When the standard experiment is repeated with the same sample of blood (back-titration method).
1	48	41	40
2	35	28	29
3	41	52	50
4	50	63	62
5	17	31	32

TABLE III.

Showing the value of formalin as a substitute for the process of Nesslerization.

Case number.	N. P. N. in mg. per cent by Nesslerization.	N. P. N. in mg. per cent by formalin method.	Case number.	N. P. N. in mg. per cent by Nesslerization.	N. P. N. in mg. per cent by formalin method.
1	19	20	22	100	101
2	36.5	36	23	37	38
3	25	26	24	40	42
4	40	40	25	32	34
5	29	28	26	21	22
6	46	44	27	42	43
7	19	20	28	28	29
8	34	36	29	26	28
9	25	26	30	34	35
10	107	110	31	19	21
11	20	18	32	58	56
12	65	63	33	40	38
13	38	40	34	40	42
14	161	164	35	32	34
15	42	40	36	20	22
16	39	41	37	90	93
17	40	39	38	26	25
18	46	44	39	36	38
19	30	30	40	68	66
20	28	29	41	38	37
21	52	50			

TABLE IV.

Results obtained by formalin method with known quantities of urea.

Case number.	Measured amount of urea, in mg.	Amount found by formalin method, in mg.
1	5	4.90
2	10	9.90
3	15	14.80
4	20	19.70
5	25	24.90
6	30	29.75
7	35	34.86
8	40	39.90

CONCLUSIONS.

From the above we come to the following conclusions:—

- (1) That the principle of using formalin in estimating nitrogen in body fluids is a sound one and that the reaction with ammonium compounds is complete; this is shown by the fact that, on using measured amounts of urea, practically the same quantities were recovered by this method.
- (2) That this method can be applied in controlling the results of back-titration in suitable cases.
- (3) That this method can be used as a substitute for the process of Nesslerization in general. The advantages, as has been already discussed, are manifold.

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TABLE I.

Rate of glycolysis in normal blood.

Case number.	MG. OF GLUCOSE IN 100 C.C. OF BLOOD GLUCOSE CONTENT AFTER							RATE OF GLYCOLYSIS IN MG. OF GLUCOSE IN 100 C.C. OF BLOOD IN						Average rate per hour, mg./100 c.c.
	Initial glucose content.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	1st hour.	2nd hour.	3rd hour.	4th hour.	5th hour.	6th hour.	
1	89	81	75	69	67	62	59	8	6	6	2	5	3	5.00
2	95	91	82	77	75	70	64	4	9	5	2	5	6	5.16
3	85	80	70	65	61	54	48	5	10	5	4	7	6	6.16
4	100	93	82	76	72	67	63	7	11	6	4	5	4	6.16
5	96	90	82	77	72	66	63	6	8	5	5	6	3	5.50
6	90	85	75	68	63	59	55	5	10	7	5	4	4	5.83
7	88	84	78	73	70	67	65	4	6	5	3	3	2	3.83
8	102	94	84	77	73	67	63	8	10	7	4	6	4	6.50
9	116	110	100	95	89	82	76	6	10	5	6	7	6	6.16
10	90	81	76	70	65	59	53	6	8	6	5	6	6	5.83
11	117	110	99	93	89	83	79	7	11	6	4	6	4	6.33
12	81	75	67	61	60	56	53	6	8	3	1	4	3	4.66
13	77	71	64	59	54	51	48	6	7	5	5	3	3	4.83
14	69	61	55	49	46	42	40	5	9	6	3	4	2	5.00
15	92	81	73	65	60	56	52	11	8	8	5	4	3	6.50
Hourly average rate of glycolysis (for first six hours).								6.2	8.7	5.66	4.06	5.0	3.93	5.56
Average velocity of percentile glycolysis (first six hours).								6.7	16.1	22.2	26.6	32.0	36.3	6.05

observed by the latter workers, the rate slowing down to 0 mg. to 15.8 mg. per hour per 100 c.c. of blood. Bhattacharyya (*loc. cit.*) found the rate in oxalated blood to be 17.4 mg. to 19 mg. per hour per 100 c.c. of blood.

The above investigations were done under different conditions. Macleod worked with normal blood rendered artificially hyperglycemic. He and also Cajori and Crouter (*loc. cit.*) studied the loss of glucose at the end of several hours and then calculated the rate of glycolysis per hour, which, though it gives the average rate, cannot however be accepted as the actual rate per hour which may vary in different periods. Thus further work was done to find :—

- (1) The rate of glycolysis per hour in normal blood.
- (2) The rate of glycolysis per hour in diabetic blood.

METHOD.

The samples of blood were all taken from Indians and without any aseptic precautions, because the period over which the blood was kept was not only short enough to preclude any significant bacterial growth but also it had been shown by Falcon-Lesses (*loc. cit.*) that works done under aseptic conditions did not alter the results. The samples were kept at 37°C. and their sugar content estimated at hourly intervals. The method used was that of Folin and Wu (1919, 1920). 0.1 per cent solution of potassium oxalate was used as the anti-coagulant.

(A) GLYCOLYSIS IN NORMAL BLOOD.

Glycolysis in 15 normal cases has been studied and the findings have been put in Table I. From a study of the table we can draw the following inferences :—

- (1) The rate of glycolysis varies between 2 mg. and 11 mg. per hour per 100 c.c. of blood in different periods, being greater in the 2nd hour in most cases, after which it slows down as more and more glucose is glycolysed.
- (2) The rate of glycolysis per hour in the different samples is found to vary between 3.83 mg. and 6.50 mg., giving an average rate of 5.56 mg. per hour, while the calculated average velocity of percentile glycolysis is found to be 6.05 mg. per hour per 100 c.c. of blood.

To find out if the initial sugar content had any effect on the rate of glycolysis, three persons were given 50 g. of glucose and samples of blood were drawn in fasting condition, and one hour after administration of glucose when the sugar content was highest, and their rate of glycolysis studied. It was found that when the initial glucose content was high the rate of glycolysis per hour was increased, there being no actual decrease in any case.

In Chart 1, the average rate of glycolysis in normal blood has been plotted and also the rate of glycolysis in one of the three normal cases cited above before and after glucose.

(B) GLYCOLYSIS IN DIABETIC BLOOD.

Glycolysis in diabetic blood has been studied in 20 established cases of diabetes and the findings have been shown in Table II. From a study of the table the following inferences can be drawn :—

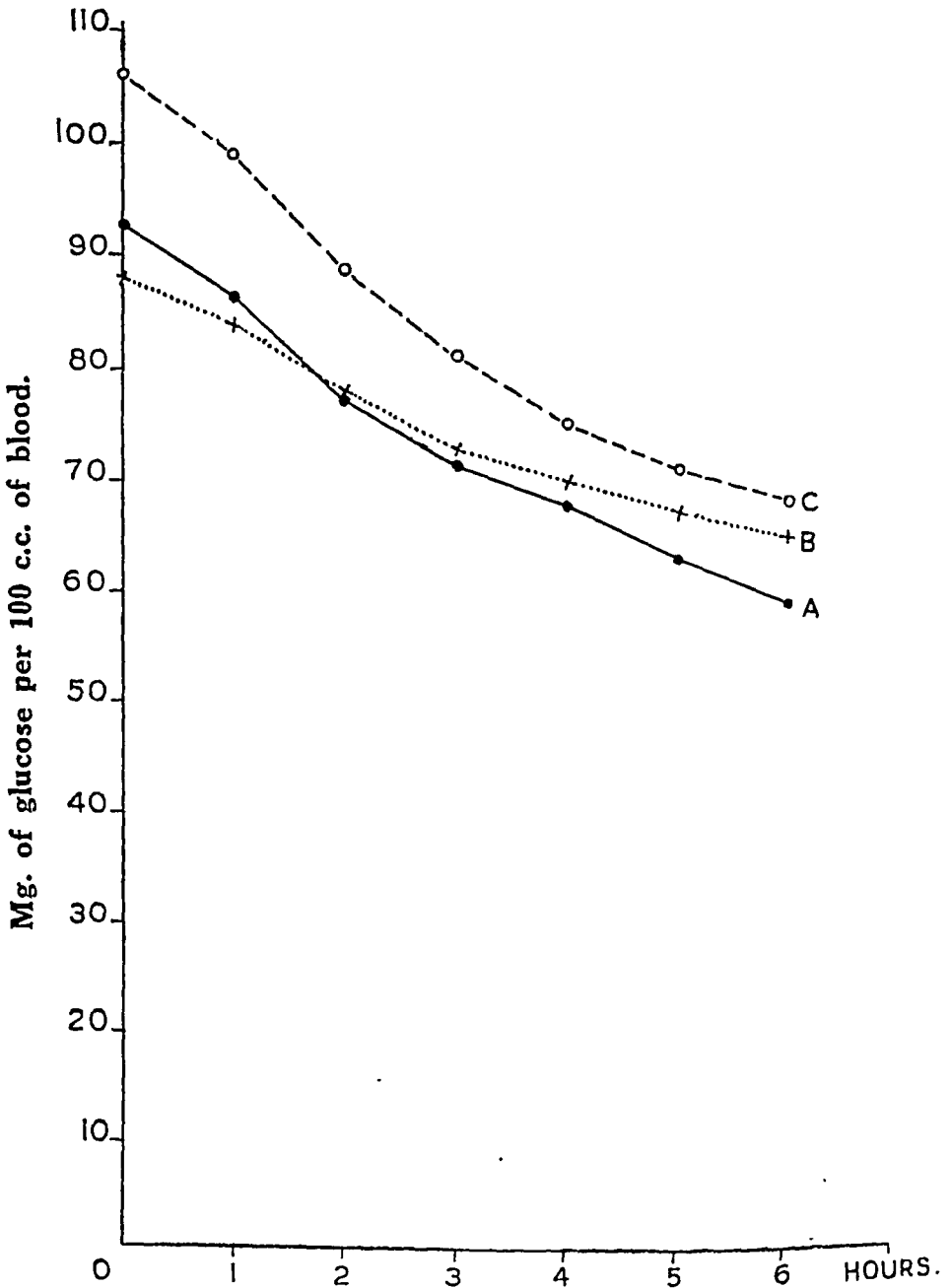
- (1) The rate of glycolysis varies from 0 mg. to 36 mg. per hour per 100 c.c. of blood. It is different in different samples. Some increase is apparent in the 2nd and 3rd hours in some of the cases, gradually slowing down in the later hours in most of the cases.
- (2) The rate of glycolysis per hour in different samples is found to vary between 4.0 mg. and 16.6 mg. per 100 c.c. of blood, giving an average rate of 11.06 mg. per hour; while the calculated average velocity of percentile glycolysis is found to be 4.61 mg. per hour per 100 c.c. of blood.

TABLE II.

Rate of glycolysis in diabetic blood.

Case number.	MG. OF GLUCOSE IN 100 C.C. OF BLOOD GLUCOSE CONTENT AFTER							RATE OF GLYCOLYSIS IN MG. GLUCOSE PER 100 C.C. OF BLOOD IN						Average rate per hour, mg./100 c.c.
	Initial glucose content.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	1st hour.	2nd hour.	3rd hour.	4th hour.	5th hour.	6th hour.	
1	228	220	202	182	166	143	131	8	18	20	16	23	12	16.1
2	190	182	170	148	133	111	102	8	12	22	15	22	9	14.6
3	256	245	236	218	208	191	178	11	9	18	10	17	13	13.0
4	360	345	323	307	293	275	250	15	22	16	14	18	15	16.6
5	190	180	167	162	156	148	142	10	13	5	6	8	6	8.0
6	200	186	178	172	161	156	152	14	8	6	11	5	4	8.0
7	240	228	205	180	168	158	146	12	23	25	12	10	12	15.6
8	210	204	190	185	178	171	166	6	14	5	7	7	5	7.3
9	320	300	288	275	260	246	236	20	12	13	15	14	10	14.0
10	166	154	141	136	136	136	126	12	13	5	0	0	10	6.6
11	228	220	212	204	192	176	162	8	8	8	12	16	14	11.0
12	192	180	170	163	155	151	149	12	10	7	8	4	2	7.1
13	200	196	182	176	169	158	153	4	14	6	7	11	5	7.8
14	260	254	234	226	224	222	220	6	20	8	2	2	2	6.6
15	356	346	330	316	308	294	276	10	16	14	8	14	18	13.3
16	300	288	272	254	244	226	216	12	16	18	10	18	10	14.0
17	240	225	215	198	185	173	164	15	10	17	13	12	9	12.6
18	222	186	182	174	161	144	129	36	4	8	13	17	15	15.5
19	210	202	192	180	170	164	156	8	10	12	10	6	8	9.0
20	200	192	190	186	176	176	176	8	2	4	10	0	0	4.0
Hourly average rate of glycolysis (for first six hours).								11.75	12.7	11.85	9.95	11.2	8.95	11.06
Average velocity of percentile glycolysis (first six hours).								4.9	10.2	15.1	19.3	24	27.7	4.61

CHART 1.



Curves of rate of glycolysis in normal blood.

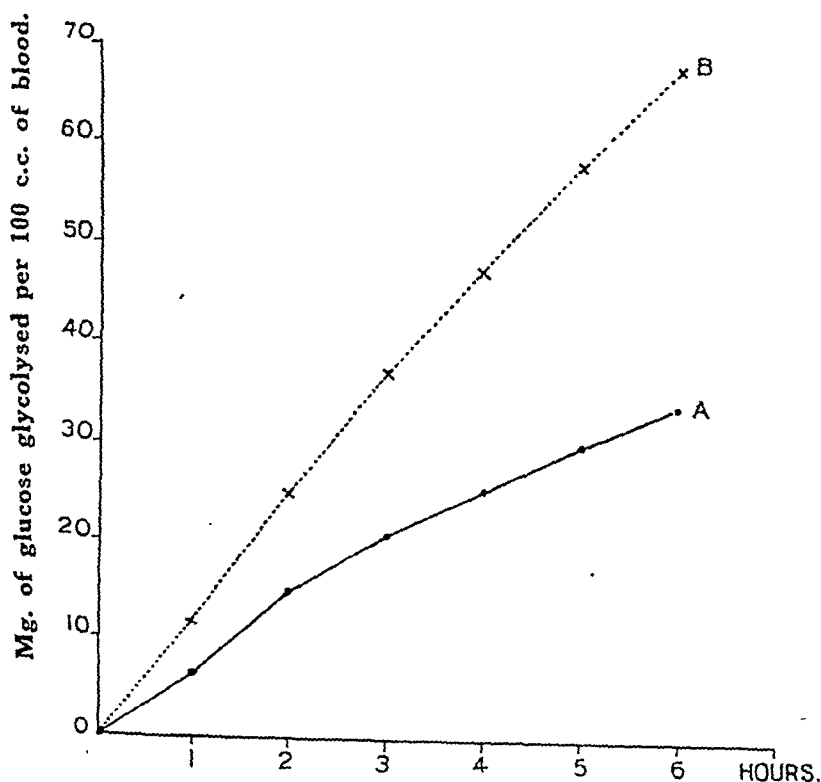
- A—Average of 15 cases (5.56 mg. per 100 c.c. glycolysed per hour).
- B—A typical case in fasting condition (3.83 mg. per 100 c.c. glycolysed per hour).
- C—The above case, blood taken 1 hour after 50 g. of glucose by mouth (6.33 mg. per 100 c.c. glycolysed per hour).

The effect of initial sugar content on the glycolysis rate was studied in three cases of diabetes by following glycolysis on fasting blood and on samples drawn one hour after administration of 50 g. of glucose. It was found, however, in contrast to the normal cases, that with increase of initial sugar concentration there was slight diminution in the rate of glycolysis.

The average rate of glycolysis in diabetic blood is plotted in Chart 2, as also the rate of glycolysis in one of the three diabetic cases cited above, before and after administration of glucose.

In Chart 3 we have shown the two average rate curves, and in Chart 4 the two percentile velocity curves of normal and diabetic cases for comparison.

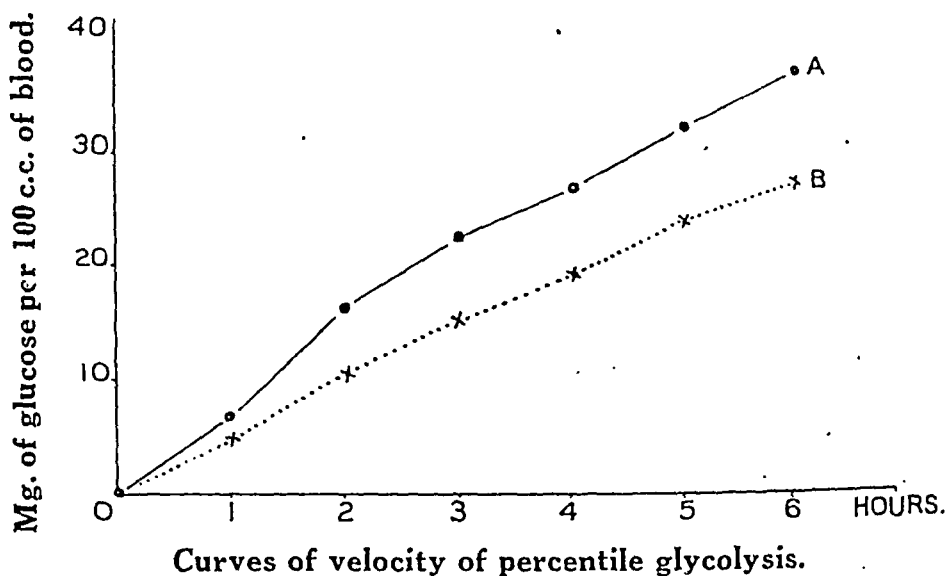
CHART 3.



Curves of total glycolysis.

A—Normal (average of 15 cases).
B—Diabetic (average of 20 cases).

CHART 4.



A—Normal (average of 15 cases).

B—Diabetic (average of 20 cases).

DISCUSSION.

On comparing the curves of glycolysis in normal and diabetic blood we find :—

The average rate of gross or total glycolysis of 11.06 mg. per hour per 100 c.c. of blood in diabetic cases is higher than that of 5.56 mg. per hour in normal blood. This does not agree with the findings of Macleod, who concludes that the increased amount of sugar in blood does not materially increase the extent of the glycolysis in a given time. But we must not forget the wide difference in the nature of experiments. Macleod worked with blood rendered hyperglycemic artificially. He also worked with blood from dogs which has a higher rate of glycolysis than that of human blood (Somoygi, 1933). We must be very careful before accepting such samples of blood as the same as drawn from the cases of actual diabetes, for possibly there is greater difference in the nature of the two samples of blood other than their sugar concentration only. Probably for these reasons our findings do not agree with his conclusion that glycolysis proceeds at the same rate in normal and diabetic blood. Schmitz and Glover (1927) also came to the same conclusion as Macleod. They stated that the initial sugar concentration of glucose within a range of 60 mg. to 250 mg. per 100 c.c. did not affect the rate of glycolysis in normal blood. They studied glycolysis in only a few cases and that also in heparinized blood. Cajori and Crouter (*loc. cit.*), however, found the rate in diabetic blood to be higher, e.g., 0 mg. to 15.8 mg. per hour per 100 c.c. compared with 5.5 mg. to 11.3 mg. per hour in normal oxalated specimens. Their finding thus agrees with that of ours in this respect. But as regards their conclusion that there is no diminution of glycolytic power in

diabetic blood while the potassium oxalate depresses profoundly the glycolysis in diabetic blood, we do not agree with them. Our finding definitely proves that there is no such depression in the absolute amount of glycolysis in oxalated blood but that there is noticeable depression of 23·8 per cent in the percentile glycolysis in diabetic blood. This can be explained by and we believe it to be due to the presence or absence in diabetic blood of other factors which control glycolysis and not due to potassium oxalate.

As regards the depression observed in the rate of glycolysis in cases of diabetes after administration of glucose we are at present unable to explain the phenomenon.

From a study of the tables it will be noticed that there is a gradual diminution in the rate of glycolysis in the later periods probably due to accumulation of the products of glycolysis—one of which at least is lactic acid (Levene and Meyer, 1912).

CONCLUSION.

1. Glycolysis takes place in normal drawn blood using potassium oxalate 0·1 per cent solution as an anti-coagulant at a rate of 3·83 mg. to 6·50 mg. (total range 2 mg. to 11 mg.) per hour per 100 c.c. of blood, giving an average rate of 5·56 mg. per hour.

2. The calculated average velocity of percentile glycolysis is 6·05 mg. per hour per 100 c.c. of normal blood.

3. In diabetic blood in oxalated samples glycolysis proceeds at a rate of 4·0 mg. to 16·6 mg. per hour per 100 c.c. of blood (total range 0 mg. to 36 mg. per hour), giving an average rate of 11·06 mg. per hour.

4. The calculated average velocity of percentile glycolysis in diabetic blood is found to be 4·61 mg. per hour per 100 c.c. of blood.

5. The average velocity of percentile glycolysis in diabetic blood per hour is found to be less by 1·44 mg. (6·05 mg. — 4·61 mg.), showing a depression of about 23·8 per cent from the normal blood.

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GLYCOLYSIS IN DRAWN BLOOD IN TWENTY-FOUR HOURS*.

BY

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FALCON-LESSES (1927) found that in heparinized blood the sugar came down to 17 mg. (Folin-Wu method) per 100 c.c. of blood in six hours when the initial sugar concentration was below 100 mg. per 100 c.c. of blood, and that further incubation even for 24 hours did not reduce the value. He deduced that all glycolysable substances had been glycolysed in six hours and the residual matter present was non-glucose copper-reducing substances. Macleod (1913) found that in defibrinated blood about half the amount of sugar disappeared in two and a half hours and in some samples practically all the sugar disappeared in 24 hours. From a study of the tables of Cajori and Crouter (1924) we find that in oxalated blood a large amount of sugar is left over even after 24 hours.

We have examined 12 samples of oxalated blood immediately after withdrawal from the vein and again after 24 hours' incubation at 37°C. and the findings have been put in Table I. From a study of the table it will be noticed that—

1. Complete glycolysis does not take place in 24 hours, the amount left over being far greater than can be accounted for as due to the presence of non-glucose copper-reducing substances.

*Read at the Physiology Section of the Silver Jubilee Session of the Indian Science Congress held jointly with the British Association for the Advancement of Science on the 3rd January 1938.

TABLE I.

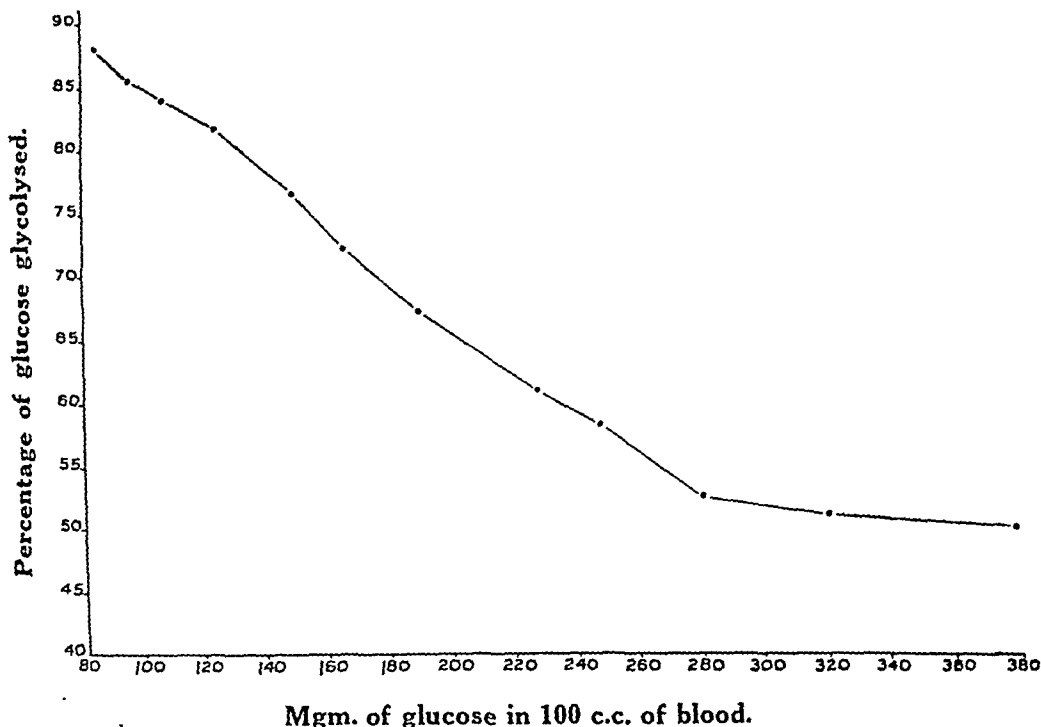
Glycolysis in blood in 24 hours.

Case number.	Initial glucose content, mg. per 100 c.c.	Glucose content after 24 hours, mg. per 100 c.c.	Amount glycolysed in 24 hours, mg. per 100 c.c.	Percentage of sugar glycolysed in 24 hours.
1	85	10	75	88.2
2	95	13	82	86.0
3	108	17	91	84.2
4	125	23	102	82.0
5	150	35	115	77.0
6	166	45	121	72.8
7	190	61	129	67.8
8	228	88	140	61.4
9	248	102	146	58.8
10	280	132	148	52.8
11	320	156	164	51.2
12	380	190	190	50.0

2. It proves that the amount of total glycolysis in 24 hours is variable according to the initial sugar concentration of the blood. This agrees to some extent with the view of Cajori and Crouter (*loc. cit.*) that the rate of glycolysis varies directly with the amount of sugar present. But it is found that though there is a greater amount of glycolysis when the initial sugar concentration is higher, yet the amount left over unglycolysed is quite large, that is, the percentage glycolysed is lesser. In fact we find the percentage of sugar glycolysed in 24 hours is more or less in an inverse ratio to the initial sugar concentration up to the level of about

280 mg. per 100 c.c. of blood, above which the extent of glycolysis remains more or less constant (see Chart).

CHART.



Showing the percentage of glucose glycolysed in 24 hours in samples of blood of varying concentrations of glucose.

This diminution in the percentage of sugar glycolysed, as we have already mentioned in another paper, is probably due to accumulation of the products of glycolysis, one of which at least is lactic acid (Levene and Meyer, 1912), which depresses the glycolysis possibly by changing the pH content. To study this we have examined several samples of blood after addition of some phosphate buffers to a portion of blood leaving the other portion unbuffered. It has been found that after 24 hours' incubation there has been a greater amount of glycolysis in the buffered portion compared to that in the unbuffered one, the increase being about 4.6 to 13.0 per cent. The difference is more marked with cases of higher sugar concentration. In cases of lower sugar content, e.g., below 100 mg. per cent, practically all the sugars were glycolysed in the buffered samples as will be seen from Table II; the amount left over is about 6 mg. to 8 mg. per 100 c.c. of blood by the Folin-Wu method, which is not reduced even on further incubation. But Falcon-Lesses found the amount to be 17 mg. per 100 c.c. of blood by the same method. Thus we could easily conclude that 6 mg. to 8 mg. per 100 c.c. of blood

found in our experiment were the non-glucose copper-reducing substances in blood and not 17 mg. The stoppage of further glycolysis in the experiments of Falcon-Lesses was probably due to accumulation of lactic acid.

TABLE II.

Glycolysis in 24 hours in unbuffered and buffered samples of blood.

Case number.	Initial sugar content, mg. per 100 c.c.	GLUCOSE CONTENT IN MG. PER 100 C.C. AFTER 24 HOURS IN		PERCENTAGE SUGAR GLYCOLYSED IN 24 HOURS IN		Per cent of excess of sugar glycolysed in buffered.
		Unbuffered.	Buffered.	Unbuffered.	Buffered.	
1	85	10	6	88.2	92.8	4.6
2	95	13	6	86.0	93.6	7.6
3	100	15	8	85.0	92.0	7.0
4	108	17	10	84.2	90.7	6.5
5	125	23	12	82.0	90.4	8.4
6	150	35	15	77.0	90.0	13.0

CONCLUSION.

1. Complete glycolysis does not take place after 24 hours' incubation in oxalated blood, a considerable portion being left over.

2. The percentage of sugar glycolysed in 24 hours is found to bear a more or less inverse ratio to the initial sugar concentration up to 280 mg. per 100 c.c. of blood.

3. Accumulation of lactic acid in blood possibly prevents further glycolysis in later periods.

4. Amount of non-glucose copper-reducing substances in blood is found to be about 6 mg. to 8 mg. per 100 c.c. of blood.

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A NEW COLORIMETRIC TEST FOR PLASMOQUINE.

BY

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(Inquiry under the Indian Research Fund Association, at the Haffkine Institute, Bombay.)

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ALTHOUGH the literature on plasmoquine is extensive, no adequate test for its determination in tissues has yet been described. Schulemann's chloranil test (Schulemann *et al.*, 1927) for plasmoquine has failed to detect minute quantities of the drug in the tissues, blood, or urine owing to the fact that chloranil develops a colour only at a concentration of 1 in 50,000 or more.

In the present work a new colorimetric test for plasmoquine is described. The test detects the presence of plasmoquine at a dilution up to 1 in a million and quantitative measurements can be made up to 1 in 200,000.

The plasmoquine employed in all our experiments was in the form of its 1 per cent solution as issued by the manufacturers in 3 c.c. ampoules for injection and all estimations were done in the form of its hydrochloric acid solution.

GENERAL DISCUSSION.

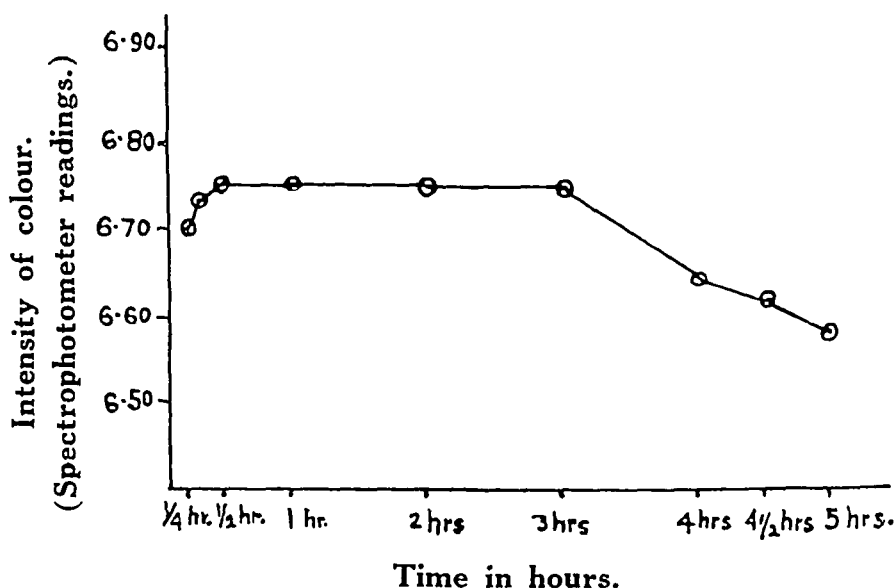
This new test is an application of Folin's phenol reagent to plasmoquine. The phenol reagent was originally meant for colorimetric determinations of tyrosine and tryptophane. There have been various modifications and improvements on it and it has been found that most of the phenol-type compounds respond to this test by developing a blue colour. The phenol reagent used for our present investigation is one with the latest improvement and is a modification by Folin and Ciocalteu (1927). For convenience we shall call the application of this test 'phenol reagent test for plasmoquine'.

When a few drops of the reagent are added to an aqueous acid solution of plasmoquine and the mixture made strongly alkaline by the addition of sodium carbonate, a blue colour develops. This colour is faint at the beginning, but in a

few minutes deepens in intensity. Generally about half an hour's time is allowed for the complete and maximum development of the colour.

The time for the maximum development of the colour has been measured by the spectrophotometer. The Graph given below represents several experiments with the spectrophotometer and it shows that in half an hour's time the colour has developed into its maximum and has been found to remain constant for three hours. At least half an hour's time, therefore, should be allowed before making any colorimetric measurements.

GRAPH.



If any precipitation occurs (as happens sometimes with plasmogquine extracted from organic tissues) when the phenol reagent and sodium carbonate have been added, the precipitate, which generally is of a colourless nature, should be centrifuged, and not filtered. The filter-papers have been found to absorb some of the colour and thus reduce the intensity, especially in cases where higher dilutions of plasmogquine are concerned.

This colour test, as has been mentioned before, can be applied to plasmogquine at a dilution up to 1 in 200,000 on a quantitative basis, when measurements are made by a Duboscq colorimeter. Beyond that dilution the colour developed is too faint for a comparison by the colorimeter. Quantitative measurements for dilutions up to 1 in a million can, however, be made by a spectrophotometer.

At every stage of this colour test, beginning from the extraction of plasmogquine from the tissues, only pure reagent chemicals must be employed. It is advisable to do a blank test by mixing in a clean test-tube the following in their order:—

- (a) About 5 c.c. of 5 per cent hydrochloric acid (required for the extraction of plasmogquine from the ether extract of the tissues),
- (b) One c.c. of 20 per cent caustic soda solution (required for adding to the tissues to be extracted),

- (c) One c.c. of phenol reagent, and
- (d) A sufficient saturated solution of sodium carbonate to make the whole alkaline.

If the reagents employed are pure, the above should give a clear and perfectly colourless solution.

It should be noted that this reagent is not specific for plasmoquine, and various substances of phenolic character in the tissues respond to it even at a considerable dilution. This difficulty is, however, removed by adding to the tissues an excess of alkali which converts all phenolic compounds into their sodium salts which are highly soluble in water and insoluble in ether. Control experiments with different tissues of various animals have given uniformly negative results when they were extracted by the above method and tested by the phenol reagent. The test can not, however, be extended to the urine, owing, probably, to the presence in urine of some reducing basic compounds extractable by ether and soluble in acid.

THE PROCEDURE

The tissue from which plasmoquine is to be extracted is first weighed and then cut into small pieces into a mortar. Sufficient (0.2 c.c. per g.) pure caustic soda solution (20 per cent) is added and then ground carefully with silver sand. The grinding must be thorough and complete so as to break the maximum number of cells possible. After grinding the mass is dried before a fan at room temperature for one to one and a half hours. The drying must not be pushed too far nor should the mass be left too moist, both of which inhibit the subsequent extraction of plasmoquine by ether. If any lumps are formed, they must be disintegrated as far as possible. The whole is then introduced into a Soxhlet apparatus of suitable size and extracted with ether for about an hour and a half. The ether extract, which is generally colourless, unless of course it has taken up some colouring matter from the tissues, as in the case of guinea-pig's and frog's liver, is washed thrice in a separating funnel with minimum quantities of 2 per cent sodium carbonate solution, after which the plasmoquine from the ether is taken up by shaking with 5 c.c. of a 5 per cent hydrochloric acid. The ether extract must not be shaken with water alone, as plasmoquine is slightly soluble in water. If about 0.02 mg. of plasmoquine is present, the acid solution will show a faint yellow colour of plasmoquine hydrochloride. The colour of plasmoquine in hydrochloric acid is not visible beyond that dilution.

Any dissolved ether is expelled by slightly warming the hydrochloric acid solution on the water-bath. To accurately measured 4 c.c. of this solution is then added 1 c.c. of the phenol reagent and the whole made strongly alkaline by adding solid sodium carbonate. The blue colour gradually develops if any plasmoquine is present. At the same time 4 c.c. of the standard solution of plasmoquine (dilution of this standard plasmoquine solution should be as near as possible to the dilution of the extract; this can be roughly judged from the depth of the yellow colour) are taken and the same quantity of phenol reagent and solid sodium carbonate added. After leaving both for about 15 minutes, the solutions are centrifuged in the case of any precipitation occurring. It is advisable to take graduated centrifuging tubes, so that any difference in volumes of the two solutions may be equalized.

After centrifuging for about five minutes the tubes are taken out and, after half an hour has elapsed, the supernatant liquid removed carefully for comparison in the colorimeter. Generally three sets of readings are recorded, of which the mean value is taken for calculation.

In the case of blood also the same procedure is to be followed, adding about 0.1 c.c. of 20 per cent caustic soda for each c.c. of blood. The blood then can either be dried on strips of filter-paper and soxhleted with ether in the usual way or it can be subjected to vigorous shaking with ether in a separating funnel *immediately* after the addition of the alkali. If the latter course is followed it is always advisable to take the blood in the separating funnel before the addition of alkali. The ether is poured out as far as possible and the blood is to be shaken twice more with fresh lots of ether. All the three lots of ether are then worked up together in the manner already described.

SOME EXPERIMENTAL RESULTS.

A few of our experiments are appended below :—

A. *In vitro.*

(1) *Blood.*

Known quantities of plasmogquine were added to separate amounts of blood and the drug extracted after different intervals; the recovered amount was then estimated by phenol reagent test. Table I represents one such experiment :—

TABLE I.

Showing the quantity of plasmogquine added to defibrinated blood and the amount recovered after extraction.

Species.	Amount of blood, in c.c.	Amount of plasmogquine added, in mg.	Time allowed to pass.	Process of extraction.	Plasmogquine found by phenol reagent, in mg.	REMARKS.
Guinea-pig	10	1	30 minutes	Soxhleting Shaking with ether.	0.93	Time factor has no influence on plasmogquine in blood of different species <i>in vitro.</i>
"	10	1	4 hours		0.92	
"	10	0.50	4 "		0.45	
"	10	0.20	4 "		0.18	
"	10	0.05	2 "		0.015	
Dog ..	10	0.05	2 "		0.043	
" ..	10	0.50	24 "	"	0.44	

Control :—

- 10 c.c. guinea-pig's blood extracted by soxhleting—negative to phenol reagent.
- 10 c.c. guinea-pig's blood extracted by shaking with ether—negative to phenol reagent.
- 10 c.c. dog's blood extracted by soxhleting—negative to phenol reagent.
- 10 c.c. dog's blood extracted by shaking with ether—negative to phenol reagent.

(2) *Blood.*

To 50 c.c. of a normal healthy dog's blood 2 c.c. of a 1 per cent solution of plasmoguin (20 mg.) were added. Lots of 10 c.c. of blood were withdrawn at different intervals and the amount of plasmoguin present estimated. The results are given in Table II:—

TABLE II.

Showing quantity of plasmoguin added to defibrinated dog's blood and the amount recovered.

Amount in c.c. of blood withdrawn from 50 c.c.	Time allowed to pass.	Process of extraction.	Plasmoguin found by phenol reagent test, in mg.	REMARKS.
10	10 minutes	Soxhleting	3.80	
10	10 "	Shaking with ether.	3.84	(a) From about 92 to 96 per cent of plasmoguin were recovered in all cases showing negligible difference with the two methods of extraction.
10	1 hour	Soxhleting	3.72	
10	2 hours	Shaking with ether.	3.68	(b) Time factor has practically no influence on plasmoguin in blood <i>in vitro</i> .
10	3 "	"	3.71	

Control:—

- (a) 10 c.c. normal dog's blood extracted by soxhleting—negative by phenol reagent test.
 (b) 10 c.c. normal dog's blood extracted by shaking with ether—negative by phenol reagent test.

B. *In vivo.*(1) *Frogs.*

(i) A frog weighing 240 g. was perfused with a solution of plasmoguin (100 c.c. of 1 in 25,000 dilution) for half an hour by the Trendelenburg method.

(ii) The tissues that were taken for extraction and the amounts of plasmoguin recovered are given in Table III. All tissues were extracted by the soxhleting process.

TABLE III.

Showing the quantity of plasmoquine recovered from various organs of the frog (R. tigrina).

Tissue.	Weight, in g.	Plasmoquine recovered, in mg.	REMARKS.
Liver	5	1.250	Total plasmoquine recovered amounted to 2.369 mg. which was about 59 per cent of the total.
Lungs	1.5	0.055	
Kidney	0.78	0.020	
Ovaries, stomach and intestines.	67	0.830	
Hind leg muscle ..	25	0.004	
Residues in the perfusing solution.	..	0.200	

(iii) The ether extracts of the ovaries, stomach, and intestines had a deep yellow colour due to some sterol compounds dissolving in the ether. This yellow substance is not, however, soluble either in alkali or acid.

(iv) In a frog weighing 220 g. half c.c. of 1 per cent solution of plasmoquine (5 mg.) was injected into the anterior lymph sac and the frog killed after three hours. Some organs were removed and their plasmoquine content estimated. The results are given in Table IV :—

TABLE IV.

Showing the quantity of plasmoquine recovered from various organs of the frog (R. tigrina).

Tissue.	Weight or volume.	Plasmoquine recovered, in mg.	REMARKS.
Blood	10 c.c.	0.200	Total plasmoquine recovered amounted to 3.277 mg. which was about 66 per cent of the amount injected.
Liver	6.5 g.	2.400	
Lungs	2.0 g.	0.290	
Kidney	0.7 g.	0.080	
Ovaries	38 g.	0.210	
Stomach and intestines.	18 g.	0.032	

(2) *Mice.*

(i) A mouse weighing 21 g. was injected hypodermically with 1 mg. of plasmoquine. It died within two hours. The whole mouse (including its excreta and hair) was cut into small pieces, ground thoroughly with alkali and sand and Soxhleted with ether.

(ii) A second mouse weighing also 21 g. was injected hypodermically with 1 mg. of plasmoquine. It died in one and a half hours. This was also similarly extracted with ether.

(iii) A third mouse weighing 20 g. was given 1 mg. of plasmoquine hypodermically. It was killed by ether after 15 minutes and extracted similarly.

The amounts of the recovered plasmoquine are shown in Table V:—

TABLE V.

Showing the amount of plasmoquine recovered from mice after hypodermic administration of 1 mg. of plasmoquine per mouse.

Mouse No.	Plasmoquine recovered, in mg.	Percentage of recovery.
1	0.622	62.2
2	0.647	64.7
3	0.807	80.7

(3) Rats.

Two rats, one weighing 385 g. (No. 1) and the other 287 g. (No. 2), were injected hypodermically each with 5 mg. of plasmoquine. Both were killed after 24 hours and their livers, lungs, etc., were extracted in the usual way. The results are shown in Tables VI and VII:—

TABLE VI.

Showing the amount of plasmoquine recovered from some organs of a rat after administration of 5 mg. of plasmoquine hypodermically.

Rat No. 1—wt. 385 g.

Tissue.	Weight, in g.	Plasmoquine recovered, in mg.	REMARKS.
Liver ..	9.5	0.281	Total recovery from these tissues amounted to 0.445 mg.
Lungs ..	1.8	0.074	
Kidney ..	1.8	0.062	
Heart ..	0.9	0.028	
Spleen ..	0.7	Trace.	

TABLE VII.

Showing the amount of plasmoquine recovered from some organs of a rat after administration of 5 mg. of plasmoquine hypodermically.

Rat No. 2—wt. 287 g.

Tissue.	Weight, in g.	Plasmoquine recovered, in mg.	REMARKS.
Liver ..	9.0	0.334	Total recovery from these tissues amounted to 0.476 mg.
Lungs ..	1.6	0.068	
Kidney ..	1.7	0.051	
Heart ..	0.8	0.023	
Spleen ..	0.7	Trace.	

(4) *Guinea-pigs.*

One guinea-pig (No. 1) weighing 590 g. was given 5 mg. of plasmoquine hypodermically and killed after 18 hours. Another (No. 2) weighing 612 g. received 20 mg. by mouth and died after 36 hours. The organs of both these guinea-pigs were removed, extracted with ether, and their plasmoquine content estimated. The results are given in Tables VIII and IX:—

TABLE VIII.

Showing the amount of plasmoquine recovered from some organs after administration of 5 mg. plasmoquine hypodermically.

Guinea-pig No. 1—wt. 590 g.

Tissue.	Weight or volume.	Plasmoquine recovered, in mg.	REMARKS.
Blood ..	5.0 c.c.	Trace.	Total plasmoquine recovered amounted to 1.580 mg. which was about 32 per cent of the amount injected.
Liver ..	17.0 g.	0.64	
Kidney ..	3.0 g.	0.15	
Spleen ..	0.7 g.	Trace.	
Lungs ..	5.0 g.	0.58	
Heart ..	2.0 g.	0.18	
Testis ..	4.0 g.	0.03	

TABLE IX.

*Showing the amount of plasmoguinine recovered from
some organs after oral administration of
20 mg. of plasmoguinine.*

Guinea-pig No. 2—wt. 612 g.

Tissue.	Weight, in g.	Plasmoguinine recovered, in mg.	REMARKS.
Liver ..	26.0	3.8	Total plasmoguinine recovered amount- ed to 5.37 mg. which was about 27 per cent of the amount given.
Kidney ..	4.5	0.76	
Spleen ..	0.8	Trace.	
Lungs ..	4.5	0.31	
Heart ..	2.0	0.24	
Testis ..	5.0	0.08	
Intestines and stomach.	30.0	0.18	

From the experimental data given above, it will be seen that the amount of plasmoguinine recovered from the tissues of animals is considerably less than the amount injected. It is true that in all experiments, except those on mice, only certain organs of the body were extracted and not the whole animal. In these experiments, however, most of the important organs were extracted and the figures of percentage recovery would have been only slightly higher, if every cell of the animal was included in the extract. The low percentage of recovery can be due either to incomplete extraction by our method or to destruction of plasmoguinine in the body tissues. *In vitro* experiments on blood show that plasmoguinine can almost completely be extracted (92 to 96 per cent) from organic fluids such as blood. Moreover, experimental data given above show that if the time allowed between the administration of the drug and extraction of tissues is longer, the recovery of plasmoguinine from the tissues is less. That this is not merely due to excretion of the drug is shown by experiments on mice. In these experiments every cell of the animal including its hair and excreta was extracted. The excretion of plasmoguinine of course could not have been much during the short period of two hours, but still the urine and faeces of the animals were included in the extracts. In these experiments if the animal was killed 15 minutes after administration of the drug the recovery was about 80 per cent, but if two hours were allowed to pass it fell down to about 63 per cent. It is evident from these results, therefore, that the low percentage of recovery is most probably due to destruction of plasmoguinine in the body tissue or to its conversion into some other compound, probably the latter.

OBEEDIENCE TO BEER'S LAW OF ABSORPTION.

The development of the blue colour with phenol reagent and plasmoguinine has been found to behave exactly in accordance with Beer's law, which clearly indicates that the colour that is developed does not undergo any chemical change, such as polymerization, dissociation, or reaction with the solute or the solvent.

Beer's law of absorption consists in the fact that when a beam of light enters a column of coloured solution, a part of it is absorbed and the light that is immersed is reduced in intensity as well as altered in colour. For most coloured solutions the following relation, known as Beer's law, between the intensity of absorption, the height of the column, and the concentration of the solution holds good. When the concentration of the solution and the height of the absorbing column vary in inverse proportion to one another, the intensity of absorption remains constant. That is, if a light from a common source is passed through two solutions of a substance of concentrations C_1 and C_2 , and the heights of the columns are h_1 and h_2 when both the solutions appear of the same depth of colour, then the concentrations and the heights stand in the following relation to one another :—

$$C_1 : C_2 = h_2 : h_1$$

$$\text{Or } C_1 \times h_1 = C_2 \times h_2 = \text{a constant.}$$

Or, as the dilutions are inversely proportional to the concentrations, for convenience of working out the formulæ with dilutions of the solutions instead of concentrations, we may express the relationship in the following manner taking d_1 as the dilution of concentration C_1 and d_2 as that of C_2 :—

$$\text{Then, } C_1 : C_2 = d_2 : d_1 = h_2 : h_1$$

$$\text{Or } d_1 \times h_2 = d_2 \times h_1.$$

The following data will show that with known dilutions plasmogquine has been found to strictly conform to the above formula :—

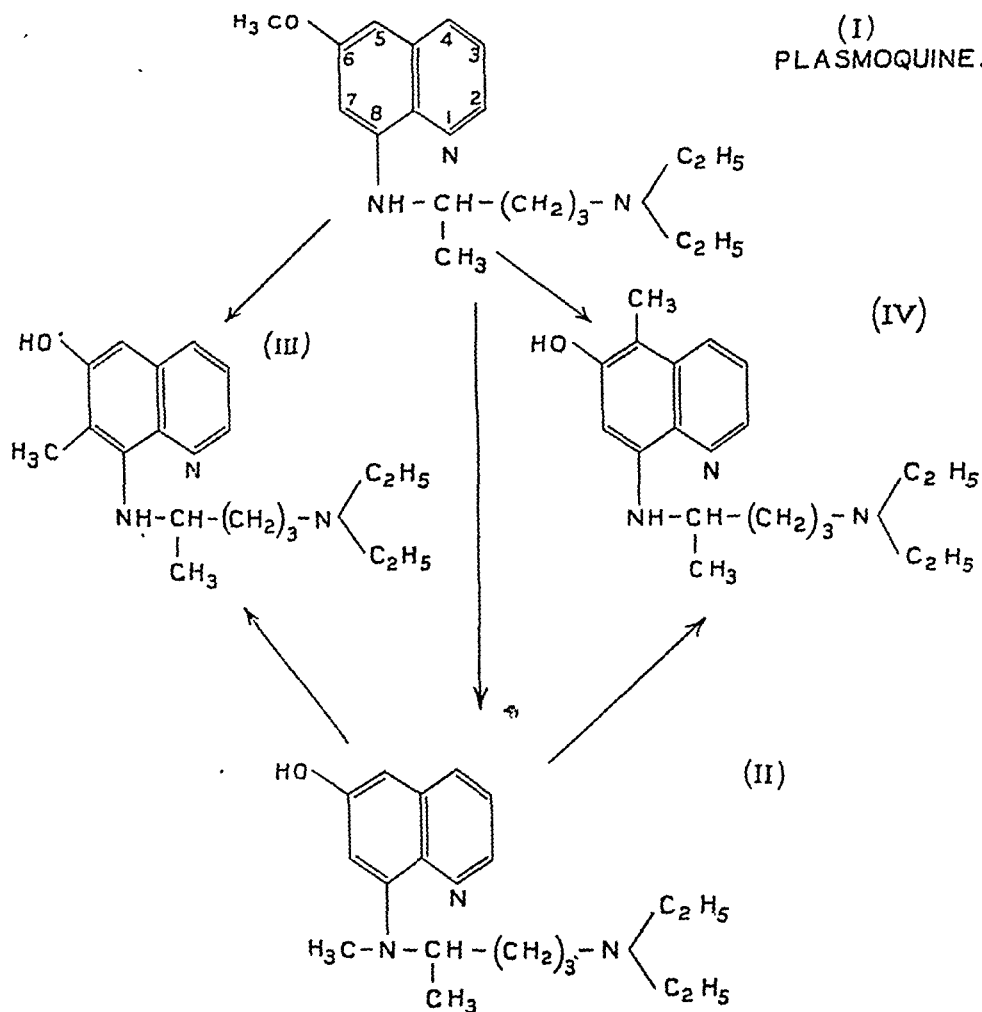
The readings have been taken in a Duboscq colorimeter and plasmogquine has been used for all standards in the form of its hydrochloride.

LEFT.		RIGHT.	
Dilution d_1 .	Reading h_1 .	Dilution d_2 .	Reading h_2 .
1 in 5,000	$\left\{ \begin{array}{c} 5 \\ 10 \\ 15 \end{array} \right.$	1 in 10,000	$\left\{ \begin{array}{c} 10 \\ 20 \\ 30 \end{array} \right.$
1 in 10,000	$\left\{ \begin{array}{c} 5 \\ 10 \\ 15 \end{array} \right.$	1 in 15,000	$\left\{ \begin{array}{c} 7.5 \\ 15 \\ 22.7 \end{array} \right.$
1 in 10,000	$\left\{ \begin{array}{c} 5 \\ 10 \\ 15 \end{array} \right.$	1 in 20,000	$\left\{ \begin{array}{c} 10 \\ 20 \\ 30 \end{array} \right.$
1 in 25,000	$\left\{ \begin{array}{c} 10 \\ 15 \\ 20 \end{array} \right.$	1 in 50,000	$\left\{ \begin{array}{c} 20 \\ 30 \\ 40 \end{array} \right.$
1 in 50,000	16	1 in 100,000	32.2
1 in 100,000	18	1 in 200,000	36

The findings of these values should leave no room for doubt about the fact that the chemical reaction taking place between the phenol reagent and plasmoquine is a thoroughly sound one as a basis for quantitative measurements.

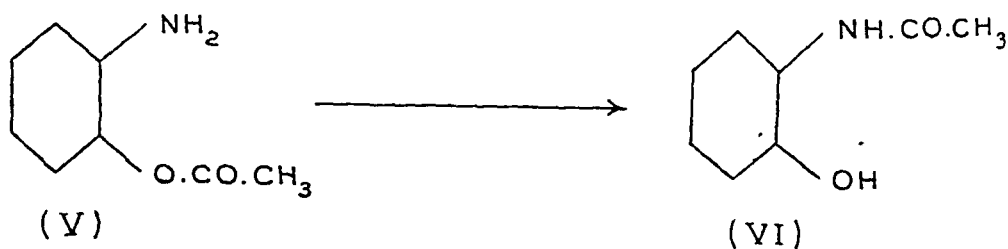
THEORY OF THE DEVELOPMENT OF THE COLOUR.

The reason why plasmoquine, whose phenolic group has been etherified and which thereby has lost its phenolic property, should respond to the phenol reagent in the development of a colour in the manner of true phenols, lies very probably in the phenomenon of an isomeric change involving migration of radicals under the influence of the phenol reagent. On this assumption the following changes in the structure of the molecule of plasmoquine may take place through the migration of the CH_3 radical and an hydrogen atom:—

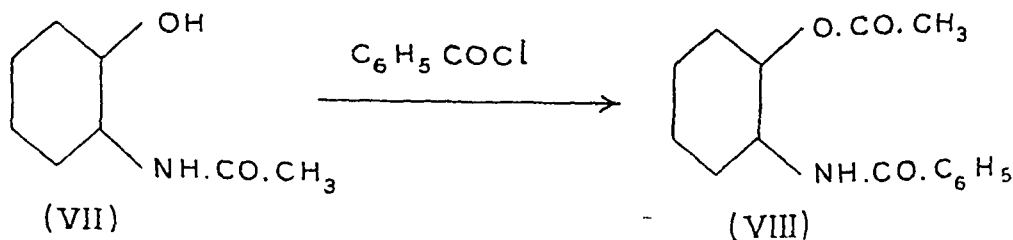


From plasmogluine, under the influence of the extremely strong oxidizing complex acids of tungsten and molybdenum, any of the three products (II), (III), and (IV) may result either directly or through the intermediary of (II), thus converting plasmogluine into a true phenol by migration of CH_3 radical and H atom. This view is confirmed by the fact that the blue colour developed by plasmogluine is decidedly considerably deeper when the addition of the reagent precedes that of the sodium carbonate than when the process is reversed. In the former case plasmogluine remains in solution in the acid medium and consequently the phenol reagent can bring about the isomeric transformation with more ease and *completely*; whereas, in the latter case, plasmogluine, being a base, is precipitated by sodium carbonate and obviously the subsequent addition of the phenol reagent does the same transformation slowly, and, what is more important, *partially*.

Examples of the isomeric change of the above type are not uncommon in chemistry. Auwers (1907-1914), Stieglitz (1909), and Ransom (1900) have found and very fully investigated the isomeric changes involving migration of radicals in which acyl radicals of aminophenols migrate from oxygen to nitrogen, if the nitrogen group is sufficiently basic, thus giving rise to a true phenol.

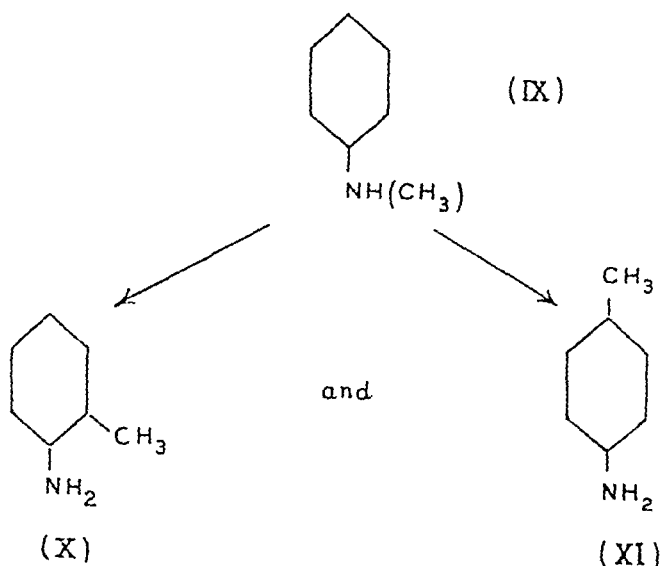


The isomeric change depicted above is almost a parallel to our assumption of the isomeric change in plasmogluine. Raiford *et al.* (1919-1925) have shown that the acetyl group in an orthoacetylaminophenol, or other aliphatic groups, is displaced by benzoyl or naphthoyl chloride in presence of alkali and the acetyl group migrates to the hydroxyl thus:—



The formation of (III) or (IV) in the case of plasmogluine may occur directly from (I) or through the intermediary of (II) in the fashion of migration of radicals from side chains to the nucleus. This latter type of conversion is most commonly experienced. It includes the reaction of Hofmann and Martius (1871), Reilly and Hickinbottom (1920), and Beckmann and Correns (1922) in which the methyl group is transferred from the nitrogen to the ortho- or para-carbon of the nucleus and the nuclear hydrogen to the nitrogen. The possibility of similar direct or indirect

migrations of radicals in plasmoguinone is more strengthened when it is now definitely known that the position para to the amino group in plasmoguinone (the 5-position in the quinoline ring) is extremely reactive and is even capable of coupling with the diazonium salts (Andersag, 1936).



Having assumed the isomeric change in the structure of plasmoguinone, the mechanism of its oxidation by phosphotungstic and phosphomolybdic acids in the phenol reagents is exactly similar to the cases of true phenols. The complex phosphotungstic and phosphomolybdic acids are extremely sensitive to reduction yielding highly coloured compounds. The chromophoric values of the reagent and chromogenic values of the substances that undergo oxidation have been at length discussed by Wu (1920), Shinohara (1935*a*), Lugg (1932), and others. It can only be mentioned here that the extent of oxidation of plasmoguinone by the complex acids in the phenol reagent is chemically a thorough and complete one and is based on an excellent quantitative relation, as has been found from the data included in the previous section.

SUMMARY.

1. A new colorimetric test has been described for plasmoguinone by the application of the phenol reagent.
2. The colour developed is strictly proportional to the concentration of plasmoguinone behaving in complete obedience to Beer's law and can be measured quantitatively up to a dilution of 1 in 200,000 although a distinct qualitative indication can be had up to a dilution of 1 in a million.
3. It takes about half an hour for the maximum development of the colour and then it remains constant for about three hours, which greatly facilitates the colorimetric measurements.

4. The test has been applied successfully to plasmoquine extracted from the tissues and blood with complete exclusion of any interfering organic agent.

5. The test could not be successfully extended to the urine owing to the presence of some interfering material in the urine. Attempts are being made to overcome this difficulty.

6. A theory of the mechanism of the development of the colour has been advanced.

7. Some experimental results have been appended.

ACKNOWLEDGMENT.

Our grateful thanks are due to Lieut.-Colonel S. S. Sokhey, I.M.S., Director, Haffkine Institute, for his keen interest in the work.

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HÆMATOLOGICAL INVESTIGATIONS IN SOUTH INDIA.

Part III.

THE MEAN RED CELL DIAMETER.

BY

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MALASSEZ (1889, quoted by Price-Jones, 1933) in France was historically the first to make systematic measurements of red blood corpuscles, but the credit of applying such measurements in clinical medicine must be given to Price-Jones, who has been working at the subject since 1910, and it is fitting that the frequency curve of the distribution of the R. B. C. diameter should be called the 'Price-Jones curve'. Though it is over a quarter of a century since such curves were shown to be useful in differentiating various types of anæmias, the method is rarely used in the practice of medicine or research, and hardly any literature exists on the subject. The explanation is that given by several workers, namely, that the technique is 'extremely laborious'. Napier and Das Gupta (1936) considered the possibility of reducing the labour involved sufficiently to bring the method within a practical sphere, but concluded that it is not a practical one for general use even in a research laboratory. The important pioneer work of Price-Jones has thus not yet borne satisfactory fruit.

Pijper (1924) devised a diffraction method of measuring the R. B. C. diameter. A simple portable apparatus (halometer) designed by Eve (1928) has been widely used by clinicians. The diffraction method does not, however, provide any indication of diameter distributions, and may give fallacious results with blood films showing extreme anisocytosis or poikilocytosis. When the method is surveyed from a critical scientific standpoint, it appears that the value attached to it by clinicians is scarcely justified. Napier and Das Gupta (*loc. cit.*) remark 'we firmly believe that it has been responsible for much mistaken diagnosis as results are seldom checked by other methods'.

During the course of an investigation of the blood in pregnant women, it was felt that a study of the R. B. C. diameter was desirable to assist in the classification of various types of anæmia. In the absence of any data on the normal red cell diameter of South Indians which could serve as a standard of comparison, the present study was undertaken.

METHOD.

The method used by us is essentially that described by Hynes and Martin (1936) for projecting blood films on a screen and taking their measurements. Certain modifications, which increase the practical usefulness of the method, have however been made.

A Bausch and Lomb euscope is employed, the source of illumination being a carbon arc lamp working on 220 volts A. C. and a current strength of 4·5 amperes. The light passing from the eyepiece of the microscope is totally reflected from the hypotenuse of a right-angled prism on to the ground-glass screen of the euscope.

The size of the projected image is calculated in the following simple manner: A 5-mm. micrometer scale is slipped into the eyepiece of the microscope. The length of the image of the micrometer scale on the screen is measured with an ordinary scale. As the dimensions of the image of the blood film projected on the screen are directly proportional to the objective used, the eyepiece remaining the same, this distance on the screen is equivalent to $5,000\mu$ divided by the magnification of the objective. From this, the distance on the screen equivalent to 1μ is easily calculated. Using the value for 1μ so obtained, a series of circles $3\cdot0\mu$ to $14\cdot0\mu$ in diameter, increasing in size by steps of $0\cdot5\mu$, are drawn in Indian ink on celluloid films. A discarded x-ray film with the silver removed is satisfactory for this purpose, and has the merit of being obtainable at no cost.

This method is in certain respects simpler than that of Hynes and Martin. In their technique the image is projected on to a horizontal ground-glass screen at a fixed magnification of 2,000 diameters by means of a microscope and a pointolite of 100 c.p. Special apparatus is required to adjust the distance between screen and microscope so as to obtain the fixed magnification. The size of the image is measured with a celluloid protractor on which circles are drawn with diameters equivalent to $2\cdot5\mu$ to $13\cdot0\mu$ at the fixed magnification of 2,000.

The blood films are made carefully so as to obtain a uniform spread of R. B. Cs. without any overlapping. The film is first stained with Leishman stain in the usual manner, then counter-stained with 1 per cent aqueous eosin for about five minutes. When dry the film is projected with an oil immersion objective and a $\times 20$ eyepiece on to the ground-glass screen, preferably in a dark room. The celluloid film with the graduated circles drawn on it is superimposed on the images of the corpuscles. By selection it is easy to find the circle that fits the image. The size of the diameter is then easily read off. The measurement of circular corpuscles is quite easy, but it is a little more difficult to deal with irregular-shaped corpuscles which occur especially in anæmic states. In measuring R. B. Cs. of this kind a circle is found on the film such that the area of the corpuscle falling outside the circle is roughly equivalent to the area of the circle which remains unfilled by the corpuscular image. With a little practice it is easy to do this even with very irregularly-shaped cells. Five hundred R. B. Cs. are measured in less than an hour's time, and from the

figures obtained the mean diameter, standard deviation, and coefficient of variation are determined.

MATERIAL.

We report here the results of observations on 25 normal healthy South Indians living in Coonoor, of whom 10 were women. The men were members of the staff of the Nutrition Research Laboratories and the Pasteur Institute, Coonoor. The women were healthy persons selected at the Lawley Hospital, Coonoor.

RESULTS.

Tables I and II give the results obtained. We have included in the tables figures giving the hæmoglobin content of the blood determined by a technique recently described (Sankaran and Rajagopal, 1938), which is in effect a modification of Bing and Baker's (1931) method. The Hb values are high, which is probably due to the fact that Coonoor is 6,000 feet above sea-level. The average R. B. C. diameter was found to be 6.85μ with a range of 6.27μ to 7.38μ . The averages for men and women were identical, being 6.85μ . The statistical data can be summarized as follows:—

Mean diameter of R. B. Cs.	..	= 6.85μ .
Range of value of mean diameter	..	= 6.27μ to 7.38μ .
Standard deviation	..	= 0.28μ .
Coefficient of variation	..	= 4.1 per cent.

TABLE I.

Statistical data obtained from 15 males.

Serial number.	Hæmoglobin in grammes per 100 c.c. blood.	R. B. C. mean diameter. (M in μ .)	Standard deviation. (σ in μ .)	Coefficient of variation in per cent.	Macrocytes. (M + 3 σ .)	Microcytes. (M - 3 σ .)
1	20.0	6.76	0.35	5.15	Nil	Nil
2	20.8	6.79	0.34	5.00	"	"
3	18.9	6.77	0.33	4.90	"	"
4	20.6	7.25	0.40	5.45	"	"
5	19.4	6.99	0.36	5.20	"	"
6	20.9	7.17	0.43	5.95	"	"
7	20.8	6.61	0.36	5.45	"	"
8	21.2	6.95	0.42	6.95	"	"
9	19.3	7.31	0.56	7.70	"	"
10	22.8	7.08	0.36	5.00	"	"
11	17.5	6.93	0.35	5.00	"	"
12	18.8	6.65	0.36	5.40	"	"
13	18.8	6.65	0.39	5.80	"	"
14	17.4	6.60	0.36	5.40	"	"
15	16.0	6.27	0.35	5.50	"	"

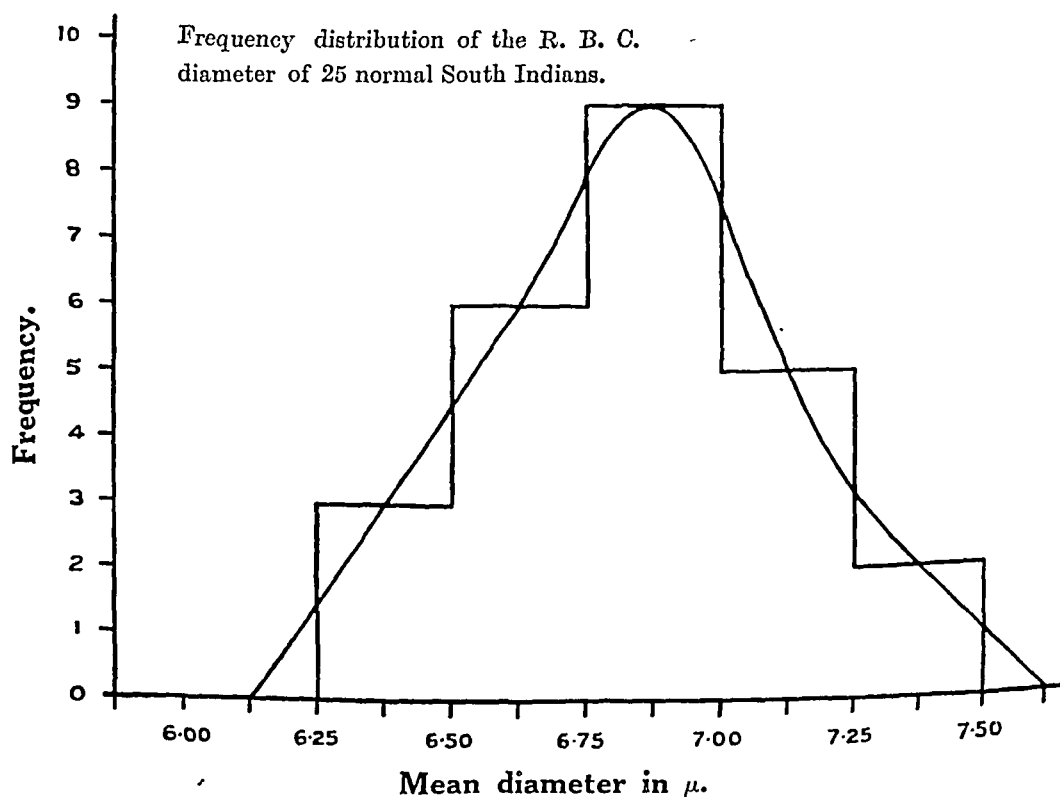
TABLE II.

Statistical data obtained from 10 females.

Serial number.	Hæmoglobin in grammes per 100 c.c. blood.	R. B. C. mean diameter. (M in μ .)	Standard deviation. (σ in μ .)	Coefficient of variation in per cent.	Macrocytes. (M + 3 σ .)	Microcytes. (M - 3 σ .)
1	19.8	6.93	0.34	4.93	Nil	Nil
2	19.2	6.69	0.39	5.85	"	"
3	17.8	7.04	0.45	6.35	"	"
4	18.6	6.87	0.52	7.55	"	"
5	20.0	7.38	0.40	5.35	"	"
6	12.6	7.19	0.54	7.50	"	"
7	18.8	6.40	0.32	5.00	"	"
8	14.5	6.49	0.39	5.95	"	"
9	17.4	6.64	0.39	5.85	"	"
10	16.8	6.85	0.39	5.75	"	"

The frequency distribution of the R. B. C. diameter of 25 normal South Indians investigated is given in the Graph. The correlation between hæmoglobin content and diameter was worked out. The coefficient was 0.32, indicating a low degree of correlation.

GRAPH.



DISCUSSION.

The relatively simple and rapid method of determining R. B. C. diameters followed here is practicable for more general adoption in clinical medicine and research. It can be carried out by any one provided with a microscope and a powerful source of light, and an hour suffices for the investigation of a blood film. The euscope could easily be replaced by an ordinary reflecting mirror fixed at an angle of 45° above the eyepiece of the microscope, throwing the image on an ordinary ground-glass plate.

It is less fatiguing to carry out the necessary measurements with a vertical than with a horizontal screen, as in Hynes and Martin's technique.

The mean R. B. C. diameters determined by us for South Indians are decidedly lower than the figure for Europeans given by Price-Jones (mean 7.202μ with a range of 6.661μ to 7.492μ). At present no reason for this difference can be suggested.

SUMMARY.

A study of the mean red cell diameter of South Indians was undertaken by a modified area method with a view to establishing normal standards. The method is simpler and less time-consuming than the original Price-Jones method. Data are presented of 25 males and females. The mean R. B. C. diameter for the group was 6.85μ , with a standard deviation of 0.28μ and a coefficient of variation of 4.1 per cent. This figure is below that given by Price-Jones for Europeans (7.202μ).

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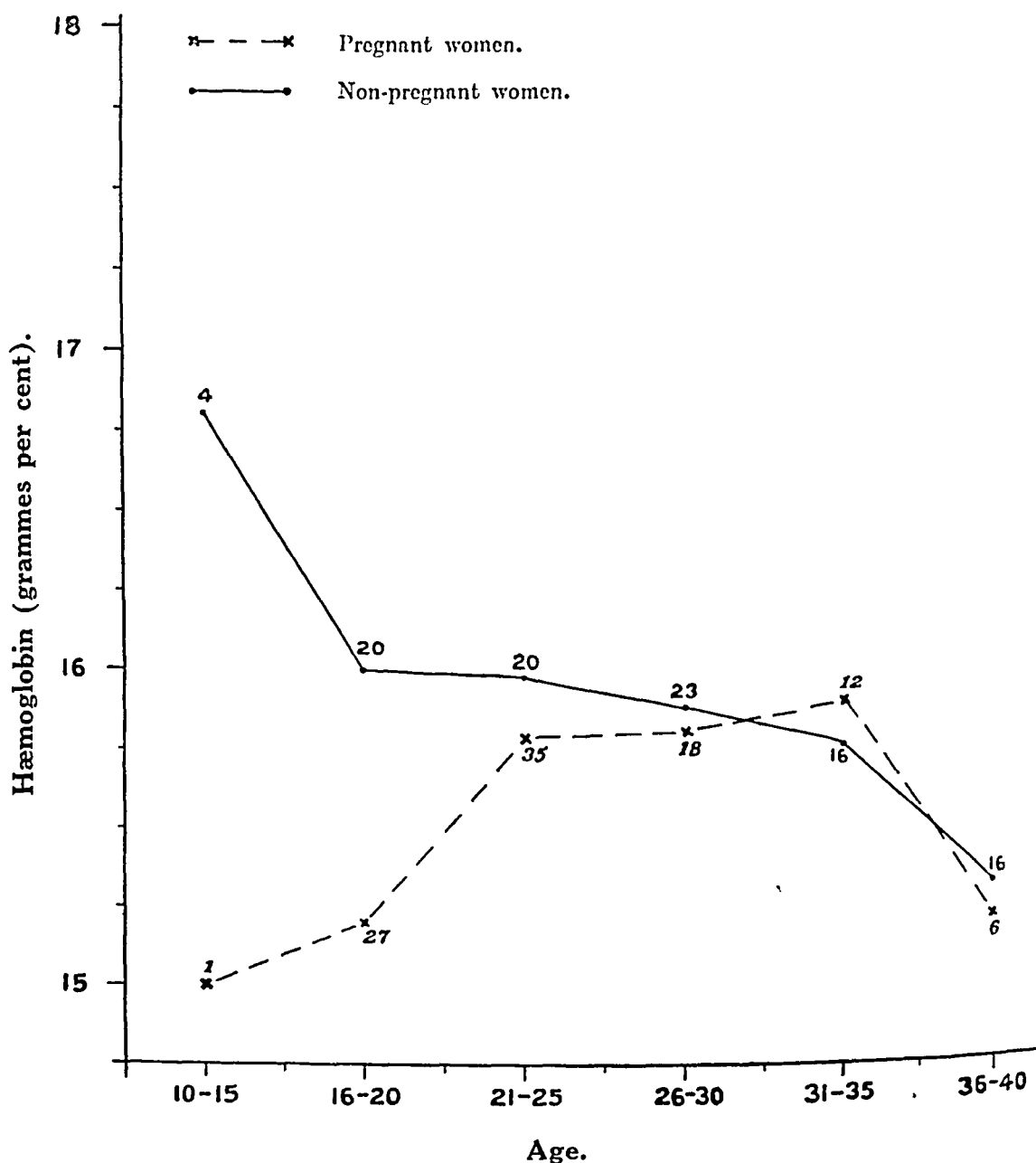


FIG. 2.—Average hæmoglobin values for pregnant and non-pregnant women in the various age groups.

BLOOD PICTURE.

The blood picture in six cases in the pregnant group showed definite evidence of anæmia indicated by the presence of macrocytes, nucleated red blood cells, polychromes, and punctate basophils. In the pregnant group, lymphocytosis was observed in 34 cases; the large mononuclears and eosinophils, except in few instances, were within normal limits.

DISCUSSION.

The advantages of Bing and Baker's modification of Wu's method used in the present investigation for the determination of hæmoglobin are fully discussed by Sankaran and Rajagopal (1938a). The method is simple and rapid; only a small quantity of blood (5 c.mm.) is required for the estimation, and a large number of estimations can be carried out within a day. The method is particularly suitable for use in field work, where a number of samples for hæmoglobin determination have to be collected in a short time.

Pure methyl alcohol is generally used in the preparation of Leishman stain. In the present investigation, methyl alcohol (methanol) technical, free from acetone, was used in the preparation of the stain with very satisfactory results; the blood films were well stained in all instances. Pure methyl alcohol is about 10 times costlier than methyl alcohol technical, and hence the latter can be used with advantage (provided it is free from acetone) in the preparation of Leishman stain in all big institutions where the stain is used in large quantities.

Hæmoglobin in pregnancy.—The hæmoglobin values obtained in this investigation suggest that in uncomplicated pregnancy there is no appreciable lowering of the hæmoglobin level among the general female population. This finding is in agreement with the observations of Napier and Das Gupta (1937) in Assam. Napier and Billimoria (1937) found that the mean Hb levels in groups of non-pregnant and pregnant women were 10.80 and 10.70 grammes per cent respectively. Fullerton (*loc. cit.*), in discussing the iron requirements of women during the reproductive life, points out that menstruation constitutes a greater demand than pregnancy. The iron that is saved by the absence of menstruation during the period of gestation and lactation is greater than that required by the demands of pregnancy.

Neither age nor parity appears to have any influence on the hæmoglobin level in both the pregnant and non-pregnant groups. Davidson, Fullerton and Campbell (1935) have shown that in women of the poorer classes in Great Britain the hæmoglobin percentage curve falls gradually throughout the reproductive life. This finding was not corroborated in the present investigation. There is no evidence to suggest that there is an appreciable lowering of the hæmoglobin level as pregnancy advances.

Several workers (Bethell, 1936; Fullerton, *loc. cit.*; Reid and Mackintosh, 1937; and others) have regarded a hæmoglobin reading below 70 per cent of the normal value as indicating anæmia. Judging by this standard, the incidence of anæmia (hæmoglobin reading below 11 grammes per cent) was no greater in the pregnant than in the non-pregnant group. According to Bethell (*loc. cit.*) 'the occurrence of hypochromic anæmia in pregnant women is not to be found primarily in the circumstances incident to gestation but should be sought in the status of the hæmopoietic mechanism prior to conception'. On the other hand, excessive loss of blood in parturition may result in hypochromic anæmia in women of the poorer classes whose diet is defective both in quality and quantity. Repeated pregnancies may thus predispose to an hypochromic anæmia.

The figures obtained in this investigation for the mean hæmoglobin in the two groups are higher than those reported for pregnant and non-pregnant women by other workers in India. This appears to be due to the effect of altitude, Coonoor being situated at about 6,000 feet above mean sea-level.

SUMMARY AND CONCLUSIONS.

1. Hæmoglobin estimations have been carried out by Bing and Baker's modification of Wu's method on 100 pregnant and 100 non-pregnant women in Coonoor, 6,000 feet above sea-level. The mean hæmoglobin values in the pregnant and non-pregnant groups were 15.52 grammes and 15.81 grammes per 100 c.c. of blood respectively.

2. No correlation was found to exist between hæmoglobin and age, parity, and duration of pregnancy.

3. In uncomplicated pregnancy the hæmoglobin level is not appreciatively below that of the general female population.

ACKNOWLEDGMENTS.

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APPENDIX A.

Hæmoglobin values of 100 pregnant women.

Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.	Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.
1	25	II	8	17.0	23	26	II	7	18.8
2	31	II	9	13.4	24	39	X	9	10.9
3	25	I	8	14.7	25	16	I	9	17.2
4	38	VIII	9	13.6	26	25	III	6	18.4
5	22	II	9	14.8	27	25	II	8	15.0
6	17	I	2½	15.5	28	28	V	8	16.5
7	22	III	9	17.0	29	20	I	6	18.3
8	18	I	9	15.3	30	22	II	7	16.1
9	16	I	8	14.2	31	14	I	7	15.0
10	25	II	9	12.0	32	23	V	6	17.9
11	18	II	4	12.2	33	30	IV	9	16.6
12	18	I	9	10.4	34	18	II	8	10.9
13	25	III	8	14.2	35	30	IV	9	16.7
14	23	III	6	12.5	36	20	III	8½	16.7
15	24	III	8	14.3	37	19	II	8	16.2
16	24	I	9	14.9	38	23	III	5	15.4
17	22	IV	2	18.3	39	34	V	9	15.4
18	20	I	7	14.2	40	21	IV	6	19.8
19	35	VIII	8	15.5	41	35	IX	8	17.2
20	21	II	9	17.0	42	25	II	9	15.2
21	18	I	3	13.4	43	25	III	8½	19.4
22	18	I	9	18.2	44	28	III	6	15.5

APPENDIX A—contd.

Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.	Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.
45	20	II	9	18.6	68	28	V	9	13.2
46	19	I	6	13.7	69	20	II	6	14.6
47	35	V	8	17.3	70	18	I	8	16.0
48	35	V	8½	21.0	71	38	VIII	8	15.1
49	16	I	9	12.7	72	30	V	7	12.7
50	17	I	8	19.1	73	36	X	2	18.5
51	24	II	6	18.1	74	20	II	4	16.2
52	22	II	7	17.3	75	18	I	3	19.4
53	30	IV	7	20.0	76	30	V	9	12.1
54	25	III	9	15.0	77	36	XI	5	18.2
55	24	II	7½	13.6	78	27	III	5	11.3
56	27	VI	9	13.2	79	25	IV	7½	12.8
57	30	IX	2	17.6	80	25	V	7	11.4
58	25	II	5	13.9	81	16	I	6	11.2
59	26	VI	7	13.2	82	34	II	7	11.6
60	18	II	5	15.5	83	22	III	5	14.1
61	24	II	3	17.4	84	35	VI	7½	15.5
62	25	III	4	14.1	85	35	III	8	17.5
63	20	I	9	14.1	86	24	II	2	19.2
64	25	II	5	8.5	87	20	I	2	16.9
65	27	IV	3	21.0	88	20	I	3	17.9
66	35	VI	8	14.9	89	36	VIII	5	14.6
67	25	III	7	12.5	90	30	VI	4	15.5

APPENDIX A—concl'd.

Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.	Serial number.	Age (in years).	Para.	Month of pregnancy.	Hæmoglobin, grammes per 100 c.c. of blood.
91	30	V	8½	16.5	96	35	VIII	7½	14.5
92	20	I	9	16.9	97	30	VII	8	17.1
93	45	V	3	18.0	98	25	III	4	18.5
94	22	III	8	11.8	99	20	II	7	12.7
95	25	II	9	18.5	100	35	VIII	2	17.4

APPENDIX B.

Hæmoglobin values of 100 non-pregnant women.

Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100 c.c. of blood.	Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100 c.c. of blood.
1	15	Nil	20.5	10	35	V	17.9
2	25	I	17.7	11	20	II	15.6
3	25	II	17.7	12	18	Nil	18.2
4	39	VI	19.7	13	20	Nil	16.4
5	43	XI	18.1	14	21	Nil	18.2
6	40	VII	20.5	15	26	I	19.2
7	40	VII	15.6	16	40	X	18.6
8	18	Nil	17.4	17	30	II	15.5
9	35	I	13.2	18	28	Nil	19.0

APPENDIX B—contd.

Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100 c.c. of blood.	Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100 c.c. of blood.
19	25	<i>Nil</i>	20.5	43	18	<i>Nil</i>	15.1
20	26	<i>Nil</i>	16.3	44	38	V	14.8
21	25	<i>Nil</i>	15.3	45	36	IV	11.1
22	30	<i>Nil</i>	14.8	46	20	I	16.6
23	40	VII	10.5	47	30	III	16.3
24	38	V	12.6	48	20	<i>Nil</i>	18.5
25	35	IV	8.3	49	28	I	13.1
26	40	<i>Nil</i>	13.8	50	25	I	15.3
27	35	<i>Nil</i>	17.4	51	15	<i>Nil</i>	13.6
28	30	V	14.7	52	20	<i>Nil</i>	13.1
29	40	II	12.3	53	38	IX	13.6
30	30	II	16.5	54	33	III	15.1
31	30	IV	13.8	55	22	I	13.5
32	26	<i>Nil</i>	15.8	56	35	VII	15.0
33	35	III	16.2	57	15	<i>Nil</i>	18.5
34	30	II	17.5	58	32	II	17.0
35	28	I	10.3	59	30	III	16.3
36	25	I	15.4	60	20	I	18.1
37	27	I	16.1	61	30	II	17.8
38	25	I	15.6	62	25	II	18.0
39	16	<i>Nil</i>	15.0	63	30	I	13.7
40	40	XVI	13.1	64	20	I	16.1
41	35	I	16.0	65	25	I	8.0
42	30	III	14.2	66	35	IX	16.2

APPENDIX B—concl'd.

Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100-c.c. of blood.	Serial number.	Age (in years).	Para.	Hæmoglobin, grammes per 100 c.c. of blood.
67	35	VIII	17.3	84	24	I	13.9
68	25	I	13.4	85	35	VII	14.9
69	20	I	14.5	86	25	I	14.4
70	20	I	15.6	87	16	I	17.6
71	30	IV	13.1	88	18	<i>Nil</i>	17.6
72	25	III	14.1	89	36	V	16.8
73	35	I	14.9	90	17	<i>Nil</i>	16.2
74	30	II	17.2	91	25	<i>Nil</i>	14.9
75	25	<i>Nil</i>	13.1	92	35	II	18.2
76	25	I	16.6	93	16	<i>Nil</i>	16.3
77	38	II	18.6	94	30	<i>Nil</i>	20.4
78	25	<i>Nil</i>	15.6	95	39	<i>Nil</i>	19.1
79	18	<i>Nil</i>	8.4	96	25	IV	18.6
80	20	I	16.1	97	15	<i>Nil</i>	14.7
81	35	V	18.5	98	28	III	16.3
82	25	VI	16.1	99	28	IV	17.4
83	40	VII	14.2	100	19	<i>Nil</i>	18.0

HÆMATOLOGICAL INVESTIGATIONS IN SOUTH INDIA.

Part V.

THE EFFECT OF ALTITUDE ON HÆMOGLOBIN CONTENT.

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IN the course of previous investigations on the hæmoglobin content of the blood of Indian men and women (Sankaran and Rajagopal, 1938*a* and *b*) it was noted that the subjects living in Coonoor in general gave a high figure which could be accounted for by the fact that Coonoor is 6,000 feet above sea-level. Since a considerable part of the inhabited area of India is plateau or mountain, it is important that the factor of altitude should not be overlooked in hæmatological studies. The present paper describes an investigation of a group of British soldiers newly arrived in the Nilgiri Hills from sea-level, which throws light on the relation between hæmoglobin content and altitude. This problem has been studied elsewhere (Haldane *et al.*, 1912; Fitzgerald, 1913; Krupski and Almasy, 1937; have made important contributions) but the authors are not aware of any similar investigations in India.

METHOD OF INVESTIGATION.

A British regiment which had been stationed in Madras (sea-level) for two years arrived in Wellington (6,000 feet above sea-level) in September 1937. A group of 38 volunteers provided the material for the investigation. All were healthy young men between 18 and 25 years, and represented a fair sample of the whole regiment. All were living under similar conditions as regards activity and diet, and the standard of general health was uniform throughout the group.

The first Hb determinations were carried out on the men three days after their arrival in the hills. It is to be regretted that estimations could not be carried out prior to the journey from Madras. Subsequently determinations were made weekly for one month and again once two months after arrival in Wellington. Blood

was taken throughout at the same time of the day. The entire group was not available for all subsequent examinations, as is indicated by gaps in Table I. The average number at examinations after the first was 28.

The hæmoglobin values of a group of British soldiers resident in Wellington for over two years were also ascertained.

Bing and Baker's modification of Wu's method, as described in an earlier paper (Sankaran and Rajagopal, 1938a), was used, results being expressed in grammes of hæmoglobin per 100 c.c. of blood.

RESULTS.

The hæmoglobin values of the group at various intervals after arrival in the hills are given in Table I, together with means, standard deviations, and coefficients of variation. The same results are shown graphically in the Chart:—

TABLE I.

Hæmoglobin values of a group of British soldiers taken at various intervals after arrival at 6,000 feet above sea-level.

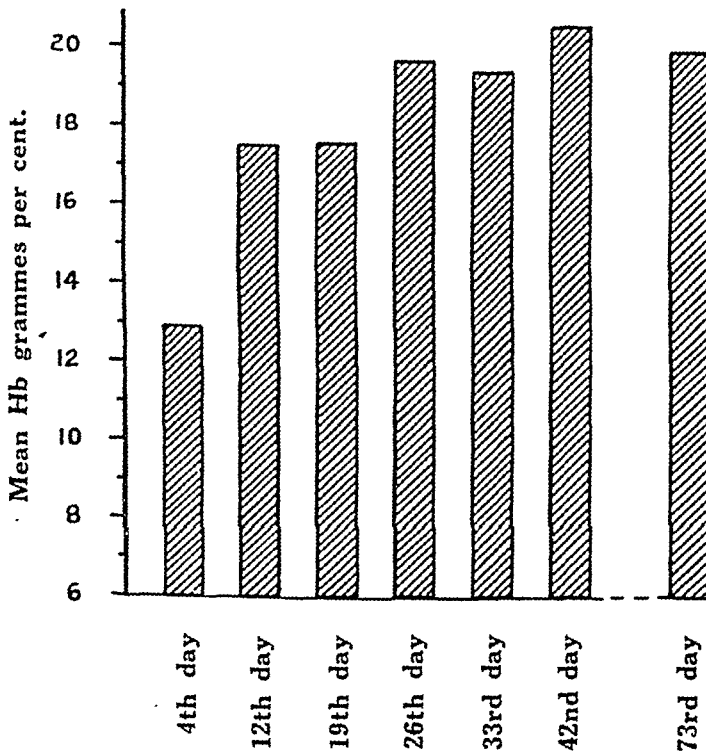
Serial number.	7-9-37	15-9-37	22-9-37	29-9-37	6-10-37	15-10-37	15-11-37
1	15.9	18.9	16.5
2	14.7	18.43	18.8	20.6	..
3	10.3	..	16.80	16.8	17.0	19.1	17.2
4	11.3	17.99	17.30	20.8	19.6
5	13.7	18.67	17.30	19.3	18.7	19.6	20.6
6	12.7	19.61	16.1	21.2	..	20.6	19.1
7	14.4	19.12	18.8	20.0	20.1	..	21.4
8	13.1	Left station.		Left station.		Left station.	
9	12.0	19.12	18.9	18.9	19.6	19.5	..
10	13.4	21.56	17.9	19.0	20.6	20.6	20.4
11	12.6	17.00	15.8
12	12.6	15.30	17.3	20.0	19.2	20.0	22.8
13	12.6	17.39	18.9	..	18.7	20.0	..
14	12.9	17.21	..	21.2	19.6	19.5	18.8
15	13.4	..	16.7	20.2	20.8	21.4	20.6
16	13.1	..	18.9	19.6	21.4	20.8	19.8
17	12.3	15.93	17.4	19.5	..	21.0	19.6
18	13.6	16.28	15.4	20.0	19.2
19	12.3	16.45	17.4	21.0	19.0	21.4	19.1
20	13.1	17.00	..	17.8	18.4	20.0	20.2
21	13.1	18.67	17.6	..	19.6	19.1	..
22	14.5	19.12	18.3	21.2	21.4	21.4	21.4
23	13.5	20.0	19.4	21.4	20.8
24	11.7	18.76	..	20.6	18.4	21.0	18.7
25	12.1	17.39	15.3	18.9	19.2	19.3	21.8
26	12.6	15.93	16.8	19.5	18.4	21.4	..
27	12.3	Left station.		Left station.		Left station.	
28	13.4	19.12	19.7	21.2	19.1	20.6	21.3
29	13.6	17.00	18.3	..	18.9	21.4	19.3
30	12.3	16.60	16.3	17.2	18.3	19.1	19.3
31	12.3	..	18.9	..	19.1

TABLE I—*conold.*

Serial number.	7-9-37	15-9-37	22-9-37	29-9-37	6-10-37	15-10-37	15-11-37
32	12.3	17.80	..	18.2	..	20.8	18.2
33	11.4	15.30	17.4	19.0	17.2	20.0	..
34	11.7	..	17.9	19.1	..	20.6	..
35	12.1	16.81	17.3	20.2	17.8	..	18.9
36	12.6	..	18.8	18.0
37	12.1	17.39	17.9	18.9	21.1	20.3	18.2
38	14.5	15.30	..	21.4	18.7	19.5	..
M =	12.84	17.46	17.48	19.56	19.23	20.38	19.65
σ =	1.03	1.57	1.11	1.21	1.10	0.81	1.50
C.V. =	8.1	9.0	6.4	6.2	5.7	4.0	7.6
	per cent	per cent	per cent	per cent	per cent	per cent	per cent

M = Mean Hb per cent. σ = Standard deviation. C.V. = Coefficient of variation.

CHART.



Increase in the hæmoglobin content of the blood of a group of soldiers on arrival at 6,000 feet above sea-level.

From Table I it will be seen that after one week the mean level rose to 17.46 g. per cent from the initial mean figure of 12.84 g.—a rise of 4.62 g. At the end of the second week the mean level was the same (17.48 g.), but after three weeks there was a further rise by 2.0 g. to 19.56 g. At the end of the fifth week the mean was 20.38 g. and one month later, at the final examination, the mean value was 19.65 g.

The mean hæmoglobin content of the blood of 125 soldiers resident for over two years at 6,000 feet above sea-level was found to be 20.38 g. per cent ($\sigma = 1.06$; C.V. = 5.2 per cent). The individual values are given in Table II :—

TABLE II.

Hæmoglobin values of a group of British soldiers after 2 years' residence at 6,000 feet above sea-level.

Serial number.	Hb, g. per cent.	Serial number.	Hb, g. per cent.	Serial number.	Hb, g. per cent.	Serial number.	Hb, g. per cent.
1	20.20	20	22.34	39	20.20	58	21.06
2	19.58	21	20.00	40	20.20	59	21.74
3	21.40	22	20.40	41	19.42	60	19.80
4	20.20	23	20.00	42	20.00	61	19.80
5	19.80	24	20.00	43	21.28	62	22.22
6	20.20	25	20.40	44	21.28	63	20.84
7	20.20	26	20.00	45	19.80	64	21.06
8	20.20	27	20.20	46	20.00	65	20.00
9	18.80	28	20.00	47	19.80	66	20.84
10	20.20	29	19.80	48	20.00	67	20.00
11	22.00	30	20.40	49	22.22	68	20.00
12	19.00	31	20.00	50	19.60	69	20.00
13	21.40	32	20.00	51	21.74	70	22.22
14	18.80	33	20.60	52	19.42	71	20.62
15	22.52	34	20.20	53	21.74	72	22.22
16	20.74	35	20.40	54	21.50	73	20.84
17	23.12	36	21.00	55	20.00	74	20.40
18	22.40	37	20.00	56	19.60	75	22.98
19	18.66	38	20.60	57	21.50	76	20.62

TABLE II—*concl'd.*

Serial number.	Hb. g. per cent.	Serial number.	Hb. g. per cent.	Serial number.	Hb. g. per cent.	Serial number.	Hb. g. per cent.
77	20.00	90	20.00	103	20.30	116	20.30
78	20.00	91	19.60	104	18.20	117	19.20
79	20.00	92	20.00	105	19.90	118	19.20
80	22.98	93	20.62	106	22.30	119	22.30
81	19.04	94	23.26	107	20.40	120	20.80
82	20.00	95	20.00	108	22.30	121	19.80
83	19.80	96	18.86	109	20.40	122	20.40
84	19.80	97	20.00	110	21.40	123	18.30
85	20.00	98	21.06	111	20.80	124	22.60
86	19.40	99	20.00	112	22.00	125	20.20
87	20.40	100	20.00	113	19.20		
88	20.62	101	18.80	114	20.00		
89	20.00	102	20.40	115	20.40		

Mean Hb = 20.38 g. per cent. Standard deviation = 1.06. Coefficient of variation = 5.2 per cent.

DISCUSSION.

In a previous investigation the mean Hb value of a group of 125 healthy young Indian men living in Madras city was found to be 16.57 g. per cent (Sankaran and Rajagopal, 1938a). The mean value in the soldiers three days after arrival in the hills from Madras was lower—12.84 g. per cent. The men were all looking pale. A rapid increase took place during the first week, and by the end of the third week a figure close to the maximum was obtained—a rise of 6.72 g. per cent in the mean level having taken place.

The level obtained within a few weeks corresponded closely with that observed in soldiers resident in the hills for over two years. From this it can be assumed that no further rise takes place after four to six weeks.

Fitzgerald (*loc. cit.*), who carried out an extensive investigation on hæmoglobin in various places in the Rocky Mountains, found that the Hb percentage varied inversely with the barometric pressure; above 8,000 feet above sea-level even as small a diminution in pressure as 50 mm. of mercury produced a rise in hæmoglobin. The effect varied considerably in different individuals. She concluded that 'for every 100 mm. fall in barometer pressure there is an increase of 10 per cent above

the amount of Hb present in the body at sea-level, the law holding true for both the sexes'. The difference in barometric pressure between Madras and Wellington is roughly 145 mm. (760 mm. to 615 mm.), the mean pressure in Wellington being about 615 mm. According to Fitzgerald's law this would involve a 15 per cent increase in Hb.

Actually the increase in the soldiers was in the neighbourhood of 50 per cent. It is possible, however, that the levels recorded on arrival from Madras were abnormally low. The mean value in young Indians in Madras was found to be 16.57 g. per cent (Sankaran and Rajagopal, 1938*a*), while for the 121 Indian subjects in Bombay it was 15.37 g. per cent (Sokhey *et al.*, 1937), the latter investigation having been carried out by determination of oxygen capacity by the van Slyke technique. If a figure between our values and those of Sokhey and his co-workers, namely 16 grammes of Hb per 100 c.c. of blood, is taken to represent 100 per cent, corresponding with average values at sea-level, then the percentage levels reached in the group of soldiers would be about 120 per cent, an increase somewhat greater than that indicated by Fitzgerald's law. Lippmann (1926) observed that at Davos (5,111 feet above sea-level) the hæmoglobin content of the blood increases by 20 per cent.

In the famous Pike's Peak Expedition, Haldane and his colleagues (*loc. cit.*) recorded an increase in Hb content to '150 per cent' at a height of 14,100 feet above sea-level. Barcroft (1914), during an expedition to the Andes, found that the Hb values of 12 natives of Cerro living at 14,200 feet above sea-level were '120 to 150 per cent'.

Haldane (1935) mentions various symptoms occurring in the members of the Pike's Peak Expedition during acclimatization; these included hyperpnœa on exertion, and in some cases nausea, vomiting, headache, and fainting. Acclimatization at the altitude of most hill stations in India (6,000 feet to 8,000 feet above sea-level) rarely involves unpleasant symptoms. Nevertheless, it is common experience that exertion is difficult during the first weeks of residence. The subjects of the present investigation stated that they found it 'difficult to get air' on first arrival in Wellington, and that they could not run 100 yards. Their capacity for exercise increased *pari passu* with the rise in hæmoglobin.

The present investigation supports the common conviction that it is wise to take things easy during the first weeks of residence in a hill station. If the rise in hæmoglobin is taken as the criterion of acclimatization, then the period required for adjustment to an altitude of 6,000 feet above sea-level is at least three weeks.

The large increase in Hb which was observed in the present investigation shows that altitude may be a factor of great importance in hæmatological investigations.

SUMMARY.

1. The hæmoglobin content of the blood of a group of British soldiers coming from sea-level to an altitude of 6,000 feet above sea-level has been investigated. Determinations were carried out at frequent intervals over a period of five weeks, with a final determination nine weeks after arrival.

2. The initial mean value was 12.84 g. per cent. At the end of three weeks a rise of 6.72 g. in the mean value was observed. No further significant rise took place after this period.

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INDEX OF AUTHORS

PAGE

A

AHMAD, B., and GUHA, R. C. Some Observations on the Experimental Production of Cataract	547
AHMAD, B. See WILSON, H. E. C.	
AYKROYD, W. R., and KRISHNAN, B. G. A Diet Survey repeated at another Season	1
AYKROYD, W. R., and WRIGHT, R. E. Red-Palm Oil in the Treatment of Human Keratomalacia	7
AYKROYD, W. R., and KRISHNAN, B. G. The Deficiencies of the South Indian Diet	367
AYKROYD, W. R., and KRISHNAN, B. G. The Treatment of Stomatitis caused by Diet Deficiency	643

B

BAGCHI, K. N., and GANGULY, H. D. Lead in Urine and Faeces	147
BARNARD, T. W. See SHORTT, H. E.	
BASU, B. C. See CHOPRA, R. N.	
BASU, C. C. Notes on a New Strain of Actinomyces obtained by Blood Culture	325
BASU, U. P. See DE, S. P., and GOSWAMI, H.	
BHADURI, N. V. See MAPLESTONE, P. A.	
BHATTACHARYYA, S. See DE, P.	
BILLIMORIA, H. S. See SOKHEY, S. S., and NAPIER, L. E.	
BROOKS, A. G. See SHORTT, H. E.	

C

CHAKRAVARTI, D. N., and TYAGI, N. Studies in ' Effects of Heat '. Part I. Biochemical and Physical Changes in Ten Cases suffering from ' Effects of Heat '	791
CHATTERJEE, C. C. See DE, P.	
CHITRE, R. G. See PATWARDHAN, V. N.	

	PAGE
CHOPRA, R. N., and BASU, B. C. A Preliminary Report on the Effect of Anti-Malarial Drugs upon the Infectivity of the Patients to the Mosquitoes	459
CHOPRA, R. N., DAS, N. N., and MUKHERJEE, S. N. Studies on the Aetiology of Epidemic Dropsy. Effect of Plasma on Tissue Culture and Chorio-Allantoic Membranes of the Chick	261
CHOPRA, R. N., and DE, N. N. A Preliminary Note on the Presence of a Histamine-like Body in the Aqueous Humour in Glaucoma associated with Epidemic Dropsy	101
CHOPRA, R. N., MUKHERJEE, S. N., and CHOWHAN, J. S. Physical Changes in Blood <i>in vivo</i> after Injection of Venom from Indian Cobra (<i>Naja naia vel tripudians</i>) into Monkeys	137
CHOPRA, R. N., and ROY, A. C. Observations on the Blood-Lipoid Changes in Opium Addicts	105
CHOPRA, R. N., and ROY, A. C. On the Estimation of Atebrin in Tissues	455
CHOWHAN, J. S. See CHOPRA, R. N.	

D

DAS, G. C. See RAY, N. N.	
DAS, N. N. See CHOPRA, R. N.	
DASTUR, N. N., and GIRI, K. V. Relative Digestibilities of Edible Fats by Castor-Seed and Pancreatic Lipases	427
DAVID, J. C. See DEY, B. B.	
DE, N. K. The Possible Use of Red-Palm Oil in supplementing the Vitamin-A Activity of common Vegetable Oils	11
DE, N. K. The Adsorption of Vitamin A and Carotene and the Isolation of Vitamin A from associated Pigments	17
DE, N. K. See MAJUMDAR, B. N.	
DE, N. K., and MAJUMDAR, B. N. Further Studies on Factors affecting the Vitamin-A Activity of Animal and Vegetable Products	857
DE, N. N. See CHOPRA, R. N.	
DE, P., and BHATTACHARYYA, S. A Comparative Study of Glycolysis in Normal and Diabetic Blood	923
DE, P., and BHATTACHARYYA, S. Glycolysis in Drawn Blood in Twenty-Four Hours	933
DE, P., and CHATTERJEE, C. C. The Rôle of Formalin in the Estimation of Nitrogen in Body Fluids	917
DE, S. P., and BASU, U. P. Action of p-Amino-benzene-sulphonamide against Streptococcal Infections in Mice	465
DE, S. S. See GHOSH, B. N.	

	PAGE
DEY, B. B., LAKSHMIKANTHAM, P., DAVID, J. C., and KRISHNASWAMI, R. Preparation, Properties, and Pharmacological Action of p-Aminobenzoyl- aminomethyl-l-hydrocotarnine	713
DEY, N. C. <i>See</i> MAPLESTONE, P. A.	
DHARMENDRA and JOHN LOWE. Attempts to Cultivate <i>M. lepræ muris</i> ..	835
DHARMENDRA. <i>See</i> JOHN LOWE.	
DIKSHIT, B. B. <i>See</i> NANDI, B. K.	

G

GALSTAUN, G. A Study of Ossification as observed in Indian Subjects ..	267
GANGULY, H. D. <i>See</i> BAGCHI, K. N.	
GHOSAL, S. C. <i>See</i> LAL, R. B., and STEWART, A. D.	
GHOSH, B. N., and DE, S. S. Investigation on the Isolation of the Neurotoxin and Hæmolysin of Cobra (<i>Naja naja</i>) Venom	779
GHOSH, B. N., and RAY, N. N. Adsorption of Antigens by Antibodies or vice versa. Part IV. The Effect of different Factors on the Floccula- tion of Concentrated Antitoxins of Tetanus and Diphtheria in the Presence of their Respective Toxins	471
GHOSH, B. N., and RAY, N. N. The Danysz Phenomena in Staphylococcal Toxin-Antitoxin Reaction	477
GIRI, K. V. The Stabilization of Vitamin C by Pyrophosphate ..	443
GIRI, K. V. The Availability of Phosphorus from Indian Foodstuffs ..	869
GIRI, K. V. <i>See</i> DASTUR, N. N.	
GOKHALE, S. K. <i>See</i> SOKHEY, S. S.	
GOSWAMI, H., and BASU, U. P. 'Available' Iron in Indian Foodstuffs ..	893
GOYAL, R. K. A Serological Study of the Actinomyces	843
GREWAL, K. S., and KOCHHAR, B. D. Vitamin-A Contents of Ghee ..	623
GUHA, R. C. <i>See</i> AHMAD, B.	

H

HARDIKAR, S. W., and MOHIUDDIN, M. G. Chemical and Pharmacological Examination of <i>Randia dumetorum</i> , N. O. <i>Rubiaceæ</i>	131
--	-----

J

JOHN LOWE and DHARMENDRA. A Study of <i>M. lepræ muris</i> in Tissue Cultures and in Chick-Embryo Medium	329
JOHN LOWE. <i>See</i> DHARMENDRA.	

	PAGE
K	
KAMALA BHAGVAT. Enzyme Method for the Estimation of Adrenaline in Suprarenal Glands	911
KOCHHAR, B. D. <i>See</i> GREWAL, K. S.	
KRISHNAN, B. G. Skimmed Milk and the Growth of School Children ..	647
KRISHNAN, B. G. <i>See</i> AYKROYD, W. R.	
KRISHNAN, B. T. Influence of Histamine and Acetyl-Choline on Intestinal Movements	95
KRISHNASWAMI, R. <i>See</i> DEY, B. B.	
L	
LAHIRI, B. N. <i>See</i> SHORTT, H. E.	
LAKSHMIKANTHAM, P. <i>See</i> DEY B. B.	
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part I. Introductory Notes and a Historical Survey	163
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part II. A Summary of Field Studies ..	177
LAL, R. B., ROY, S. C., and GHOSAL, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part III. Some Laboratory Studies on Rice as the Ætiological Agent	215
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part IV. Experiments to test the Validity of Infection Theory in a Semi-Isolated Community	233
LAL, R. B., and ROY, S. C. Investigations into the Epidemiology of Epidemic Dropsy. Part V. Feeding Experiments on Human Volunteers	239
LEAGUE OF NATIONS' Fifth International Course in Malariology ..	829
LINTON, R. W., SEAL, S. C., and MITRA, B. N. Chemical and Serological Variation in Single-Cell Cultures of <i>Vibrio cholerae</i> and Related Organisms	575
LINTON, R. W., SHRIVASTAVA, D. L., and SEAL, S. C. Studies on the Specific Polysaccharides of the Vibrios. Part I. The Effect of the Growth-Medium	569
M	
MAHAL, H. S. Studies on Blood Choline Esterase	703
MAJUMDAR, B. N., and DE, N. K. The Oxalic-Acid Content of some Indian Foodstuffs	671
MAJUMDAR, B. N. <i>See</i> WILSON, H. E. C., and DE, N. K.	

MALANDKAR, M. A. See SOKHEY, S. S.	
MANNADI NAYAR, A. S. See SHORTT, H. E.	
MAPLESTONE, P. A., and BHADURI, N. V. <i>Tania solium</i> and <i>Cysticercus cellulosæ</i> in India	155
MAPLESTONE, P. A., and DEX, N. C. Laboratory Tests on the Fungistatic and Fungicidal Effect of various Substances	603
MCGUIRE, J. P. See SHORTT, H. E.	
MCRBERT, G. R. See SHORTT, H. E.	
MEHTA, D. R. Studies on Typhus in the Simla Hills. Part VIII. Ectoparasites of Rats and Shrews with Special Reference to their Possible Rôle in the Transmission of Typhus	353
MEHTA, D. R. See SMITH, R. O. A.	
MENON, K. P. See SHORTT, H. E.	
MITRA, B. N. See LINTON, R. W.	
MOHUDDIN, M. G. See HARDIKAR, S. W.	
MUKERJI, B. Action on Smooth Muscles of some Ephedrine-like Amines ..	113
MUKHERJEE, S. N. See CHOPRA, R. N.	

N

NANDI, B. K., and DIKSHIT, B. B. A New Colorimetric Test for Plasmoquine	937
NAPIER, L. E., and BILLIMORIA, H. S. Haematological Studies in Indians. Part VIII. Analysis of the Haematological Findings in 52 Cases of Anæmia amongst Pregnant Tea-garden Coolie Women	529
NARASIMHAMURTHY, G. The Nitrogen Complex of Indian Foodstuffs. Condiments: Part II. Chillies (<i>Capsicum annum</i>) and Coriander Seeds (<i>Coriandrum sativum</i>)	863
NARASIMHAMURTHY, G., and RANGANATHAN, S. The Nitrogen Complex of Indian Foodstuffs. Condiments: Part I. Black Pepper (<i>Piper nigrum</i>)	373
NARINDRA SINGH. See SHOURIE, K. L., and PAL, R. K.	

P

PAL, R. K., and NARINDRA SINGH. The Effect on Rats of Supplementing a South Indian Diet with Calcium and Phosphorus	693
PANDIT, C. G., SANJIVA RAO, R., and SHORTT, H. E. Response of the Chorio-Allantoic Membrane of the Developing Chick-Embryo to Inoculation with various Substances with Special Reference to <i>B. proteus</i> X19	595
PANDIT, C. G. See VENKATRAMAN, K. V.	
PANDIT, S. R. See SHORTT, H. E.	

	PAGE
PATWARDHAN, V. N., and CHITRE, R. G. Studies in Calcium and Phosphorus Metabolism. Part I. The Calcium and Phosphorus Content of the Soft Tissues of Normal Rats	633
R	
RADHAKRISHNA RAO, M. V. Studies on Vitamin-A Deficiency. Part II. Histopathology of the Skin in Human Keratomalacia	39
RADHAKRISHNA RAO, M. V. Studies on Vitamin-A Deficiency. Part III. Lesions of the Peripheral Nervous System	661
RADHAKRISHNA RAO, M. V. Hæmatological Investigations in South India. Part IV. Hæmoglobin in Normal Pregnancy	957
RADHAKRISHNA RAO, M. V. See SANKARAN, G.	
RAJAGOPAL, K. See SANKARAN, G.	
RANGANATHAN, S. The 'Available' Iron in some common Indian Foodstuffs, determined by the α, α' -dipyridine Method	677
RANGANATHAN, S., and SANKARAN, G. The Excretion of Vitamin C by Human Beings in South India	29
RANGANATHAN, S., SUNDARARAJAN, A. R., and SWAMINATHAN, M. Survey of the Nutritive Value of Indian Foodstuffs. Part II. Changes in Chemical Composition brought about by Cooking	45
RANGANATHAN, S. See SANKARAN, G., and NARASIMHAMURTHY, G.	
RAY, N. N., and DAS, G. C. The Immunization of Horses for the Production of High-Titre Tetanus Antitoxin	617
RAY, N. N. See GHOSH, B. N.	
ROY, A. C. See CHOPRA, R. N.	
ROY, G. K. See WILSON, H. E. C.	
ROY, N. C. See WILSON, H. E. C.	
ROY, S. C. See LAL, R. B.	
RUDRA, M. N. Studies in Vitamin C. The Effect of Cooking and Storage on the Vitamin-C Contents of Foodstuffs	89
S	
SANJIVA RAO, R. See PANDIT, C. G.	
SANKARAN, G., and RADHAKRISHNA RAO, M. V. Hæmatological Investigations in South India. Part III. The Mean Red Cell Diameter	951
SANKARAN, G., and RAJAGOPAL, K. Hæmatological Investigations in South India. Part I. The Estimation of Hæmoglobin	741
SANKARAN, G., and RAJAGOPAL, K. Hæmatological Investigations in South India. Part II. The Effect of the Administration of Iron on the Hæmoglobin Level in Indian Girls and Young Women	753

	PAGE
SANKARAN, G., and RAJAGOPAL, K. Hematological Investigations in South India. Part V. The Effect of Altitude on Hemoglobin Content ..	971
SANKARAN, G., and RANGANATHAN, S. Migration of Adrenaline in an Electrical Field	81
SANKARAN, G. See RANGANATHAN, S.	
SEAL, S. C. See LINTON, R. W.	
SHORTT, H. E., MCGUIRE, J. P., BROOKS, A. G., STEPHENS, E. D., and LAHIRI, B. N. The Relative Immunizing Values of different Forms of Antirabic Vaccine, and the Duration of Immunity in Experimentally Immunized Animals	483
SHORTT, H. E., McROBERTS, G. R., BARNARD, T. W., and MANNADI NAYAR, A. S. Endemic Fluorosis in the Madras Presidency ..	553
SHORTT, H. E., PANDIT, S. R., MENON, K. P., and SWAMINATH, C. S. The Absence of Effective Immunity after Cure of Protozoal Infections ..	763
SHORTT, H. E., and SWAMINATH, C. S. The Viability of <i>Leishmania donovani</i> excreted in the Nasal Mucus in Indian Kala-azar	341
SHORTT, H. E. See SWAMINATH, C. S., and PANDIT, C. G.	
SHOURIE, K. L., and NARINDRA SINGH. A Diet Survey of a Group of Sikh Soldiers	655
SHRIVASTAVA, D. L. See LINTON, R. W.	
SINTON J. A. The Successful Transmission of Cutaneous Leishmaniasis by Inoculation to Man from a Natural Lesion occurring on a Dog in India	787
SMITH, R. O. A., and MEHTA, D. R. Studies on Typhus in the Simla Hills. Part VII. Attempts to Isolate a Strain of XK Typhus from Wild Rats	345
SOKHEY, S. S., GOKHALE, S. K., MALANDKAR, M. A., and BILLIMORIA, H. S. Red Cells, Hemoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part I. Normal Indian Men: A Study based on the Examination of 121 Men	505
SOKHEY, S. S., GOKHALE, S. K., MALANDKAR, M. A., and BILLIMORIA, H. S. Red Cells, Hemoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part II. Normal Indian Women: A Study based on the Examination of 101 Women	723
STEPHENS, E. D. See SHORTT, H. E.	
STEWART, A. D., and GHOSAL, S. C. On the Value of Wilson and Blair's Bismuth Sulphite Medium in the Isolation of <i>Bact. typhosum</i> from River Water	591
SUNDARARAJAN, A. R. Phytin-Phosphorus Content of Indian Foodstuffs ..	685
SUNDARARAJAN, A. R. See RANGANATHAN, S.	

- CHEMICAL**: changes in foodstuffs by cooking, 45; examination of *Randia dumetorum*, 131; variation in single-cell cultures of *V. cholera*, 575.
- CHICK**: effect of plasma on chorio-allantoic membrane of, in epidemic dropsy, 261; embryo, *M. lepræ muris* in medium of, 329, response of chorio-allantoic membrane of, to *B. proteus* X19, 595.
- CHILDREN**, skimmed milk and growth of school, 647.
- CHILLIES**, nitrogen complex of, 863.
- CHOLERA** epidemic in a rural area in S. India, 585. *See also V. cholera*.
- CHOLINE**: blood choline esterase, 703; acetylcholine, influence of, on intestinal movements, 95.
- CHORIO-ALLANTOIC** membrane of chick, effect of plasma on, in epidemic dropsy, 261; response of, to *B. proteus* X19, 595.
- COBRA** venom, isolation of neurotoxin and hæmolysin of, 779; physical changes in blood *in vivo* after injection of Indian, 137.
- COEFFICIENT**, *see proteus*.
- COLORIMETRIC** test for plasmoquine, 937.
- COLOUR INDEX**, 505, 723.
- COMPLEX**, *see* nitrogen complex, condiments.
- CONDIMENTS**, nitrogen complex of, 373, 863.
- COOKING**, effect of, on vitamin-C content of foodstuffs, 89; changes in chemical composition in foodstuffs by, 45.
- COOLIE WOMEN**, anaemia amongst pregnant tea-garden, 529.
- CORIANDER** seeds, nitrogen complex of, 863.
- CORIANDRUM SATIVUM*, *see* coriander seeds.
- CULTIVATION** of *M. lepræ muris*, 835.
- CULTURE**, *see* blood, single-cell, tissue.
- CURE**, *see* protozoal infections.
- CUTANEOUS LEISHMANIASIS**, *see* leishmaniasis.
- CYSTICERCUS CELLULOSÆ* in India, 155.
- DANYSZ PHENOMENA** in staphylococcal toxin-antitoxin reactions, 477.
- DEFICIENCY**: of S. Indian diet, 367; treatment of stomatitis caused by diet, 643; vitamin-A, 39, 661.
- DERMATITIS**, *see* vitamin B₆.
- DIABETIC** blood, glycolysis in, 923.
- DIAMETER**, *see* red cells.
- DIET**: S. Indian, deficiencies of, 367, effect on rats of supplementing, by calcium and phosphorus, 693; treatment of stomatitis caused by deficiency of, 643; survey, of Sikh soldiers, 655, repeated at another season, 1. *See also* foodstuffs, nutrition, vitamin.
- DIGESTIBILITY**: of edible fats by castor-seed and pancreatic lipases, 427; coefficient of proteins, 847.
- DIPHTHERIA**, flocculation of antitoxin of, 471.
- DIPYRIDINE** method, *see* α, α' -dipyridine method.
- DOG**, *see* cutaneous leishmaniasis.
- DONOVANI*, *see* *Leishmania donovani*.
- DROPSY**, epidemic: ætiology of, 261; feeding experiments on volunteers in, 239; field studies in, 177; histamine-like body in aqueous humour in glaucoma associated with, 101; historical survey of, 163; infection theory in, 233; rice as ætiological agent in, 215.
- DRUGS**, *see* antimalarial.
- DUMETORUM*, *see* *Randia dumetorum*.
- ECTOPARASITES** of rats and shrews in transmission of typhus, 353.
- EDIBLE FATS**, *see* fats.
- 'EFFECTS OF HEAT'**, biochemical and physical changes in cases suffering from, 791.
- ELECTRICAL FIELD**, migration of adrenaline in an, 81.
- EMBRYO**, *see* chick-embryo.
- ENDEMIC**, *see* fluorosis.
- ENZYME**, *see* adrenaline.
- EPHEDRINE-LIKE AMINES**, action on smooth muscles of, 113.
- EPIDEMIC**, *see* cholera, dropsy.
- EPIDEMIOLOGY**, *see* dropsy.
- ESTERASE**, blood choline, 703.
- ESTIMATION**: of adrenaline in suprarenal glands, 911; of atebirin in tissues, 455; of hæmoglobin, 741; of nitrogen in body fluids by formalin, 917.
- EXCRETION**, *see* vitamin C.
- FÆCES**, lead in, 147.
- FATS**, edible, digestibilities of, by castor-seed and pancreatic lipases, 427.
- FEEDING** experiments, *see* dropsy.
- FIELD STUDIES**, *see* dropsy. **FIELD**, electrical, 81.
- FLAVINE** content of Indian foodstuffs, 879.
- FLOCCULATION**, *see* antitoxins.
- FLUIDS**, *see* body fluids.
- FLUOROSIS**, endemic, in Madras Presidency, 553.
- FOODSTUFFS**: Indian: availability of phosphorus from, 869; 'available' iron in, 677, 893; changes in chemical composition of, by cooking, 45; effect of cooking and storage on vitamin-C content of, 89; flavine and vitamin-B₆ content of, 879; nitrogen complex of

- (condiments), 373, 863; nutritive value of, 45; oxalic-acid content of, 671; phytin-phosphorus content of, 685; proteins of, in nutrition, 57, 381, 399; protein and non-protein nitrogenous constituents in, 847. *See also* diet, nutrition, vitamin.
- FORMALIN** in estimation of nitrogen in body fluids, 917.
- FUNGICIDAL** effect of substances, 603.
- FUNGISTATIC** effect of substances, 603.
- GHEE**, vitamin-A content of, 623.
- GIBSONI**, *see* *Babesia gibsoni*.
- GIRLS**, Indian, effect of iron on hæmoglobin level in, 753.
- GLANDS**, *see* suprarenal glands.
- GLAUCOMA**, histamine-like body in aqueous humour in, 101.
- GLYCOLYSIS**, in normal and diabetic blood, 923; in drawn blood, 933.
- GROWTH**: biological values of proteins of foodstuffs measured by, of young rats, 57; skimmed milk in, of school children, 647.
- METHOD**, supplementary values of proteins of foodstuffs studied by, 399. **MEDIUM**, effect of, in polysaccharides of vibrios, 569.
- HÆMATOLOGICAL** investigations in S. India, 741, 753, 951, 957, 971; findings in cases of anæmia in pregnant tea-garden Indian coolie women, 529.
- HÆMOGLOBIN**: 505, 723; effect of iron on hæmoglobin level in Indian girls and young women, 753; effect of altitude on hæmoglobin content, 971; estimation of, 741; in normal pregnancy, 957.
- HÆMOLYSIN** of cobra venom, isolation of, 779.
- HEAT**, *see* 'effects of heat'.
- HIGH-TITRE**, *see* tetanus antitoxin.
- HISTAMINE**, influence of, on intestinal movements, 95; like body in aqueous humour in glaucoma associated with epidemic dropsy, 101.
- HISTOPATHOLOGY**, *see* keratomalacia.
- HISTORICAL** survey, *see* dropsy.
- HORSES**, *see* antitoxin.
- HUMOUR**, aqueous, histamine-like body in, in glaucoma, 101.
- HYDROCOTARNINE**, *see* p-aminobenzoyl, etc.
- HYGIENE**, *see* London School of Hygiene and Tropical Medicine.
- IMMUNITY**, after cure of protozoal infections, absence of, 763; duration of antirabic, in immunized animals, 483. **IMMUNIZATION** of horses for high titre tetanus antitoxin, 617. **IMMUNIZING VALUES** of different forms of antirabic vaccine, 483.
- INDEX STANDARDS** (colour, saturation, and volume) for normal Indian men, 505, for normal Indian women, 723.
- INDIA**: *Taricha* and *Cyphopterus* cellulosus in, 155; transmission of cutaneous leishmaniasis to man from dog in, 787. **SOUTH INDIA**: cholera epidemic in a rural area in, 585; excretion of vitamin C by human beings in, 29; hæmatological investigations in, 741, 753, 951, 957, 971.
- INDIAN**: boys: basal metabolism of, in Calcutta, 901; cobra venom: physical changes in blood after injection of, 137; foodstuffs: 'available' iron in, 677, 893, availability of phosphorus from, 869, flavin and vitamin-B, content of, 879, nitrogen complex of (condiments), 373, 863, nutritive value of, 45, oxalic-acid content of, 671, phytin-phosphorus content of, 685; girls and young women: effect of iron on hæmoglobin level in, 753; kala-azar: viability of *L. donovani* excreted in nasal mucus in, 341; subjects: classification in, 267; standard indices for normal, men, 505, women, 723.
- INDIANS**: hæmatological studies in, 529; **SOUTH INDIAN**: diet: deficiencies of, 367; supplementing with calcium and phosphorus, 693.
- INFECTION**: protozoal, absence of immunity after cure of, 763; streptococcal, in mice, action of p-amino-benzene-sulphonamide, 465; theory in epidemic dropsy, 233; **INFECTIVITY** of patients to mosquitoes, effect of antimalarial drugs upon, 459.
- INOCULATION**, *see* *B. proteus* X19, cutaneous leishmaniasis.
- INTERNATIONAL** Course in Malariology, League of Nations', 829.
- INTESTINAL MOVEMENTS**, influence of histamine and acetyl-choline on, 95.
- IRON**, 'available', in Indian foodstuffs, 677, 893; effect of administration of, on hæmoglobin level in Indian girls and young women, 753.
- ISOLATION**, *see* *Bact. typhosum*, hæmolysis, neurotoxin, strain of XK typhus, vitamin A.
- KALA-AZAR** (Indian), viability of *L. donovani* excreted in nasal mucus in, 341.
- KERATOMALACIA** (human), histopathology of skin in, 39; red-palm oil in treatment of, 7.
- LABORATORY** studies on rice as ætiological agent in epidemic dropsy, 215; tests on fungistatic and fungicidal effect of substances, 603.
- LEAD** in urine and fæces, 147.
- LEAGUE OF NATIONS'** 5th International Course in Malariology, 829.
- LEISHMANIA DONOVANI**, viability of, in nasal mucus in kala-azar, 341.

LEISHMANIASIS, cutaneous, transmission of, by inoculation to man from lesion on a dog in India, 787.

LEPRÆ MURIS, see *M. lepræ muris*.

LESIONS, see cutaneous leishmaniasis, peripheral nervous system.

LIPASES, pancreatic, digestibilities of edible fats by, 427.

LIPOID, see blood-lipoid.

LIVER autolysates, transformation of carotene into vitamin A in, 85.

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE, University Readership in Medical Parasitology at, 833.

M. LEPRÆ MURIS, attempts to cultivate, 835; in tissue culture and in chick-embryo medium, 329.

MADRAS PRESIDENCY, endemic fluorosis in, 553.

MALARIAL, see antimalarial.

MALARIOLOGY, League of Nations' 5th International Course in, 829.

MAN, transmission of cutaneous leishmaniasis to, 787. MEN, normal Indian, index standards in, 505.

MEAN red cell diameter, 951.

MEDICAL PARASITOLOGY, London University Readership in, 833.

MEDIUM, effect of growth medium in polysaccharides of vibrios, 569; *M. lepræ muris* in chick-embryo, 329; Wilson and Blair's bismuth sulphite, 591.

MEMBRANE, chorio-allantoic of chick, effect of plasma on, in epidemic dropsy, 261; response of, to inoculation with *B. proteus* X19, 595.

METABOLISM, basal, in Indian boys in Calcutta, 901; calcium and phosphorus, 633.

METHYL, see p-aminobenzoyl, etc.

MICE, action of p-amino-benzene-sulphonamide against streptococcal infections in, 465.

MIGRATION, see adrenaline.

MILK, see skimmed milk.

MONKEYS, see cobra venom.

MOSQUITOES, see antimalarial drugs.

MUCUS, nasal, see kala-azar, *L. donovani*.

MURIS, see *M. lepræ muris*.

MUSCLES, smooth, action of ephedrine-like amines on, 113.

NAIA NAIA VEL TRIPUDIANS, see cobra venom.

NAJA NAJA, see cobra venom.

NASAL MUCUS, see kala-azar, *L. donovani*.

NERVOUS SYSTEM, peripheral, lesions of, in vitamin-A deficiency, 661.

NEUROTOXIN of cobra venom, isolation of, 779.

NIGRUM, see *Piper nigrum*.

NITROGEN: complex of Indian foodstuffs, condiments, 373, 863; rôle of formalin in estimation of, in body fluids, 917. NITROGENOUS constituents in foodstuffs, 847.

NON-PROTEIN, see protein.

NORMAL: blood, glycolysis in, 923; Indian men and women, standard indices in, 505, 723; pregnancy, hæmoglobin in, 957; rats, calcium and phosphorus content of soft tissues of, 633.

NUTRITION, proteins of foodstuffs in, 57, 381, 399. NUTRITIVE VALUE of Indian foodstuffs, survey of, 45.

NUTS, biological protein value of, 381.

'OGAWA', see *V. cholerae*.

OIL, red-palm, in treatment of keratomalacia, 7, use of, in supplementing vitamin-A activity of common vegetable oils, 11; seeds, biological protein value of, 381.

OPIUM addicts, blood-lipoid changes in, 105.

ORGANISMS related to *V. cholerae*, single-cell cultures of, 575.

OSSIFICATION in Indian subjects, 267.

OXALIC-ACID content of Indian foodstuffs, 671.

p-AMINO-BENZENE-SULPHONAMIDE, action of, against streptococcal infections in mice, 465.

p-AMINOBENZOYL-AMINOMETHYL-L-HYDROCOTARNINE, preparation, properties, and pharmacological action of, 713.

PALM OIL, see red-palm oil.

PANCREATIC LIPASES, digestibilities of edible fats by, 427.

PARASITOLOGY, see Medical Parasitology.

PATIENTS, see antimalarial drugs.

PEPPER, see black pepper.

PERIPHERAL NERVOUS SYSTEM, see nervous system.

PHARMACOLOGICAL action of p-aminobenzoyl-aminomethyl-l-hydrocotarnine, 713; examination of *Randia dumetorum*, 131.

PHOSPHORUS: metabolism, content of soft tissues of normal rats, 633; availability of, from Indian foodstuffs, 869; effect of supplementing a S. Indian diet with, 693. See also phytin-phosphorus.

PHYSICAL CHANGES, see blood, 'effects of heat'.

PHYTIN-PHOSPHORUS content of Indian foodstuffs, 685.

- PIGMENT** associated with vitamin A and carotene, isolation of vitamin A from, 17.
- PIPER NIGRUM**, nitrogen complex of, 373.
- PLASMA**, effect of, on tissue culture and chorio-allantoic membrane of chick in epidemic dropsy, 261.
- PLASMOQUINE**, a new colorimetric test for, 937.
- POLYSACCHARIDES**, *see* vibrios.
- PREGNANT, PREGNANCY**, *see* hematological studies.
- PREPARATION and PROPERTIES** of p-aminobenzoyl-aminomethyl-1-hydrocotarnine, 713.
- PROTEINS**, of foodstuffs, biological value of, of cereals, pulses, and skimmed milk powder, by growth of rats, 57; of pulses, oil-seeds, nuts, and skimmed milk, by balance-sheet method, 381; supplementary values, 399; relative amounts of protein and non-protein nitrogenous constituents and their significance in determination of digestibility co-efficient of protein, 847. *See* also nutrition.
- PROTEUS**, *see* *B. proteus* X19.
- PROTOZOAL** infections, absence of immunity after cure of, 763.
- PULSES**, biological protein value of, 57, 381.
- PYROPHOSPHATE**, stabilization of vitamin C by, 443.
- RABIES**, *see* antirabic.
- RANDIA DUMETORUM**, *N. O. RUBIACEÆ*, chemistry and pharmacology of, 131.
- RATS**: calcium and phosphorus content of soft tissues of normal, 633; ectoparasites of, in transmission of typhus, 353; effect on, of supplementing a S. Indian diet with calcium and phosphorus, 693; strain of XK typhus from wild, 345; supplementary protein values of foodstuffs, by growth of young, 57, 399, by balance-sheet method using adult, 399.
- READERSHIP**, *see* London University Readership in Medical Parasitology.
- RED CELLS**, 505, 723; mean red cell diameter, 951.
- RED-PALM OIL** in treatment of human keratomalacia, 7; use of, in supplementing vitamin-A activity of common vegetable oils, 11.
- RICE**, *see* dropsy.
- RIVER WATER**, isolation of *Bact. typhosum* from, 591.
- RUBIACEÆ** (*N. O.*), *see* *Randia dumetorum*.
- RURAL AREA** in S. India, cholera epidemic in, 585.
- SATIVUM** (*Coriandrum*), *see* coriander seeds.
- SATURATION INDEX**, 505, 723.
- SCHOOL** children, skimmed milk and growth of, 647. *See* also London School of Hygiene, etc.
- SEEDS**, *see* castor, coriander.
- SEROLOGICAL** study of actinomyces, 843; variation in single-cell cultures of *V. cholerae*, etc., 575.
- SHIREWS**, ectoparasites of, in transmission of typhus, 353.
- SIKH SOLDIERS**, diet survey of, 655.
- SIMLA HILLS**, typhus in, 345, 353.
- SINGLE-CELL** cultures of *V. cholerae*, etc., variation in, 575.
- SKIMMED MILK**, and growth of school children, 647; biological protein value of, 381, (powder), 57.
- SKIN**, *see* keratomalacia.
- SOLDIERS**, *see* Sikh soldiers.
- SOLIUM**, *see* *Tania solium*.
- SOUTH INDIA, SOUTH INDIAN**, *see* India, Indian.
- STABILIZATION**, *see* vitamin C.
- STANDARDS**, *see* index standards.
- STAPHYLOCOCCAL** infections in mice, action of p-amino-benzene-sulphonamide against, 465.
- STAPHYLOCOCCAL** toxin-antitoxin reaction, Danyasz phenomena in, 477.
- STOMATITIS** due to diet deficiency, treatment of, 643.
- STORAGE**, effect of, on vitamin-A content of foodstuffs, 89.
- STRAINS**, *see* actinomyces, typhus.
- SULPHITE**, *see* bismuth sulphite medium.
- SULPHONAMIDE**, *see* p-amino-benzene, etc.
- SUPPLEMENTARY (PROTEIN) VALUES** of foodstuffs, 399.
- SUPRARENAL GLANDS**, enzyme method for estimation of adrenaline in, 911.
- SURVEY**: diet, at another season, 1, of Sikh soldiers, 655; historical, of epidemic disease, 163; of nutritive value of foodstuffs, 45.
- TANIA SOLIUM** in India, 157.
- TEA-GARDEN**, *see* anaemia.
- TETANUS**, *see* antitoxin.
- TISSUE**: calcium and phosphorus content of, of rats, 633; culture, *M. typhi* on, 322; effect of plasma on, in epidemic disease, 261; estimation of atebirin in, 575.
- TOXIN**, *see* antitoxin.
- TRANSFORMATION**, *see* actinomyces, typhus.
- TRANSMISSION**, *see* actinomyces, typhus.
- TREATMENT**, *see* keratomalacia, dropsy.
- TRIPUDIANS**, *see* castor.

TROPICAL MEDICINE, *see* London School of Hygiene, etc.

TYPHOSUM, *see* *Bact. typhosum*.

TYPHUS in Simla Hills, isolation of NK strain from wild rats, 345; ectoparasites of rats and shrews in transmission of, 353.

URINE, lead in, 147.

VECTOR, *see* arthropod vector.

VEGETABLE: products, vitamin-A activity of, 857; oils, vitamin-A activity of, when supplemented by red-palm oil, 11. *See also* foodstuffs.

VENOM: *Naia naia* *vel* *tripudians* (Indian cobra), physical changes in blood *in vivo* after injection of, into monkeys, 137; *Naja naja* (cobra), isolation of neurotoxin and hæmolysin of, 779.

VIABILITY, *see* *L. donovani*.

VIBRIO CHOLERÆ: chemical and serological variation in single-cell cultures of, 575; epidemic in a rural area in S. India by 'Ogawa' type of, 585.

VIBRIOS, polysaccharides of, 569.

VITAMIN: A: deficiency, 39, 661; contents of ghee, 623; transformation of carotene into, in liver autolysates, 85; activity, of vegetable oils, red-palm oil in supplementing, of vegetable oils, 11, of animal and vegetable products factors affecting, 857; adsorption and isolation of, from pigments associated with carotene 17.

B₆: (anti-dermatitis) contents of foodstuffs 879.

C: stabilization of, by pyrophosphate, 443; excretion of, by human beings in S. India, 29; effect of cooking and storage on, 89.

VOLUME INDEX, 505, 723.

WATER, *see* river water.

WILD RATS, strain of NK typhus from, 345.

WILSON and BLAIR'S bismuth sulphite medium in isolation of *Bact. typhosum* from river water, 591.

WOMEN, Indian, administration of iron on hæmoglobin level in, 753; index standards in normal, 723; anæmia in pregnant tea-garden coolie, 529.

